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The Influence Exerted by Desoxycorticosterone Acetate upon the Production of Adrenal Tumors in Gonadectomized Mice

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Nodular hyperplasia or adenomas have been shown to develop in the adrenal glands of certain strains of mice, notably CBA, DBA, BALB, NH, and A, after the removal of their gonads (5, 7, 8, 13, 21). These changes were usually accompanied by the elaboration of either estrogenic or androgenic hormones or both (3, 6). In CE mice, carcinomas developed in 100 per cent of the females and in 97 per cent of the males (4, 14, 16, 18, 22). Such tumors have been successfully transplanted to mice of the same or F_1 hybrid strains (20).

Stimulation of the uterus, the vagina, and the mammary glands (4, 6, 14, 15, 17), and the appearance of cornified cells in vaginal smears (7) were accepted as evidence that estrogenic hormones had been produced; and changes in the submaxillary glands and in Bowman's capsules in the kidneys and stimulation of seminal vesicles and prostate glands were accepted as evidence that androgens had been elaborated (4, 6, 14, 15, 17).

Diethylstilbestrol (19), testosterone propionate, androstanediol, or 17-methyl androstanediol (11) inhibited the adrenal changes which developed upon gonadectomy, whereas progesterone and other steroids were found to be ineffective.

Cortical adenomas were produced in oöphorectomized C3H mice when the animals were placed upon a calorie-restricted diet, but estrogens were not elaborated (1). Gonadotrophin (10) or adrenocorticotrophin (9) did not incite estrogenic stimulation in calorie-restricted animals. Hyperplasia of the pituitary glands, with changes in the basophilic cells, developed in certain mice observed for periods of time up to 26 months after gonadectomy (2).

Woolley and Chute administered various steroids to gonadectomized CE mice and concluded that desoxycorticosterone acetate, provided in pellet form, had not restricted the development of the adrenal tumors.

This report covers the results of an investigation wherein we have studied the changes which developed in the adrenal glands of gonadectomized CE mice with and without the influence exerted by desoxycorticosterone acetate when it was given daily by subcutaneous injection.

METHODS

Female and male CE mice were used. All mice were gonadectomized when they were 1 month old. Desoxycorticosterone acetate, 5 mg. per cubic centimeter of peanut oil, was given subcutaneously to some groups of gonadectomized animals in amounts equivalent to 0.1 mg. of the hormone daily. Injections were begun on the day of operation and were given daily for the ensuing 6 months (Table 1). Vaginal smears were prepared at frequent intervals. At the termination of the experiment, the adrenal glands, uterus, vagina, seminal vesicles, submaxillary glands, thymus, kidneys, liver, spleen, and heart were removed, fixed in formalin, and prepared for histologic study. Most of the adrenal glands were sectioned serially in order to study all portions of the cortices. In some instances in which the tumors were visible grossly, a limited number of sections were prepared for study.

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RESULTS

Gonadectomized female mice of the CE strain.—Fifteen of 18 gonadectomized, untreated female CE mice survived for the duration of the period of observation. Carcinoma developed in both adrenals of 11 mice and in one adrenal of each of the other 4 mice (Table 2). Marked variations in the sizes of these glands were recorded. Of the 30 glands observed, 8 weighed 5.0 mg. or less, 15 weighed from 5.0 to 10.0 mg., 4 weighed from 10.0 to 20.0 mg., and 3 glands weighed respectively 201, 254, and 327 mg. Yellowish nodules, ranging in size from pinpoints to those covering a large surface of the adrenal, developed in the smaller glands. The larger adrenals (Fig. 1) consisted entirely of actively growing carcinomatous cells which had involved the entire gland and had completely destroyed the normal architecture.

TABLE 1

SUMMARY OF EXPERIMENTAL PROCEDURES ON CE MICE

Number of animals	Sex	Treatment	Duration of treatment	Animals surviving at termination of experiment: No.	Animals surviving at termination of experiment: Per cent	Time after gonadectomy when killed* (months)
18	F	None	None	15	83	8½
11	F	DOCA† 0.1 mg.	6 months	7	64	8½
14	M	None	None	12	86	9
17	M	DOCA 0.1 mg.	6 months	12	71	9

* All animals gonadectomized at 1 month of age.

† Desoxycorticosterone acetate.

TABLE 2

INCIDENCE OF CORTICAL CARCINOMAS IN GONADECTOMIZED FEMALE CE MICE

	Controls, no treatment	Given desoxycorticosterone acetate (0.1 mg. daily)
Number gonadectomized	18	11
Postoperative deaths*	3	4
Number completing experiment	15	7
Number developing carcinoma	15†	1
Per cent developing carcinoma‡	100	14

* Not due to operative failure but to unknown factors during the 6 months' injection period.

† Eleven mice had bilateral carcinoma of the adrenal cortex. In four the observed lesions were unilateral, but sections of the other adrenal in two of these four mice were not available for study.

‡ Per cent of number completing experiment.

We have used the term "carcinoma *in situ*" to describe the pathologic state wherein the pleomorphic elements were restricted to focal regions in the hyperplastic adrenal cortex. These tumors did not have well defined capsules surrounding them (Fig. 2), but represented a transitional state in the development of a carcinoma—that is, an early carcinomatous change. True carcinomas of the cortex were identified by cellular pleomorphism, active mitosis, hyperchromatic nuclei, and considerable invasiveness (Fig. 3). These nodules, usually arising in the zona glomerulosa, extended into the deeper zona fasciculata or invaded the peripheral capsule.

The vaginas of these fifteen gonadectomized CE mice showed varying degrees of epithelial stimulation. In eight of them there was marked stimulation (Fig. 4), while in seven the vaginal epithelium was atrophic, as in the castrate state. In some mice the epithelium was well cornified, while in others it had the appearance of pseudo-mucification resembling that of diestrus (Fig. 5). Uteri showed evidence of estrogenic stimulation, were usually cystic, and showed increased glandular development and an increase in the vascular bed.

The submaxillary glands in ten of these fifteen female mice were of the male type, wherein the epithelium of the intralobular ducts was composed of columnar cells, with nuclei located at the base of the cells. The submaxillary glands of one were of the female type, wherein nuclei were centrally located (Fig. 6), and those of three mice were of a type intermediate between the two. The glands of one mouse were not available for study.

TABLE 3

PATHOLOGIC CHANGES ARISING IN ADRENAL CORTICES OF GONADECTOMIZED FEMALE CE MICE

	Controls, no treatment: Adrenals Right	Controls, no treatment: Adrenals Left	Given desoxycorticosterone acetate (0.1 mg. daily): Adrenals Right	Given desoxycorticosterone acetate (0.1 mg. daily): Adrenals Left
Carcinoma	9	9	0	0
Carcinoma *in situ*	4	4	1	1
Nodular hyperplasia	0	2	3	5
Cells, Type B	0	0	1	1
Normal gland	0	0	1	0
No section available	2	0	1	0
Total	15	15	7	7

In male mice and in castrates given testosterone, the parietal layers of Bowman's capsules in the kidneys were composed largely of cuboidal epithelium; and in three of the fifteen gonadectomized female mice in which adrenal carcinomas developed, the male type of epithelium was identified in Bowman's capsules of the kidneys.

The influence exerted by desoxycorticosterone acetate on the adrenal pathologic changes of castrate CE female mice.—Desoxycorticosterone acetate, in 0.1 mg. daily amounts, was given daily to seven gonadectomized female mice for 6 months following castration (Tables 2 and 3). All were killed 10 weeks later. There were no enlarged adrenal glands, and only one of the fourteen adrenals contained nodules which were visible grossly. "Carcinoma *in situ*" was identified in both adrenals in

this one animal, so that an incidence of carcinoma of 14 per cent occurred in this small group of gonadectomized mice which had received the hormone. In five of the seven mice, nodular hyperplasia occurred in one or in both adrenals (Fig. 7); and in one mouse the glands were normal, except for the appearance of cells which have been designated as "Type B" cells by Dr. Woolley. The incidence of cortical carcinomas in untreated castrate mice and in castrate mice given desoxycorticosterone acetate is shown in Table 2, and the classification of the pathologic changes encountered in the adrenal glands of these test animals is shown in Table 3. The uterus and the vagina of all castrate female mice given desoxycorticosterone acetate were atrophic, and the submaxillary glands were all of the female or castrate type (Fig. 6). It seems obvious that neither estrogens nor androgens were elaborated in these castrate mice given desoxycorticosterone acetate.

Male mice of the CE strain.—An adrenal carcinoma developed in but one of twelve untreated castrate male mice which survived the postoperative period. A diagnosis of "carcinoma *in situ*" was made in its left adrenal. In nine of twelve animals nodular hyperplasia developed, and in one of the twelve mice "Type B" cells were identified in both adrenals. Seminal vesicles and prostate glands were atrophic in ten of the twelve mice, and they were slightly stimulated in the other two.

The influence exerted by desoxycorticosterone acetate on the adrenal pathologic changes in castrate CE male mice.—Twelve gonadectomized male CE mice were given 0.1 mg. of the hormone in peanut oil daily for 6 months and then were killed 3 months thereafter. Of these twelve, carcinoma did not appear in any adrenal gland. In five of them, nodular hyperplasia developed either in one or in both adrenals; in three of them, "Type B" cells developed either in one or in both adrenals, and in four the glands were entirely normal. Seminal vesicles and prostate glands were atrophic, and the submaxillary gland architecture was of the female or castrate type. Although carcinoma did not appear in any adrenal gland of any castrate male CE mouse given the hormone, nodular hyperplasia and "Type B" cells did develop in many of them; so that the conclusion seems warranted that in castrate males desoxycorticosterone acetate was without significant effect.

COMMENT

These data show that carcinoma had developed in the adrenal glands of all gonadectomized untreated female CE mice. When desoxycorticosterone acetate was given to such castrate females, at a level of 0.1 mg. daily, true carcinomas did not develop, although one animal with a diagnosis of "carcinoma *in situ*" in both adrenal glands was encountered. Nodular hyperplasia did occur in five of the seven mice, and "Type B" cells were identified. Our data are thus at some variance with those of Woolley and Chute, who gave this hormone to gonadectomized CE mice over a period of months and concluded that desoxycorticosterone acetate had not restricted the development of adrenal tumors in their animals.

We have used the same strain of mice, which was originally obtained from the Bar Harbor Laboratories, but the technics employed varied in some ways from those employed by Woolley and Chute. Time of gonadectomy may have been a factor. Woolley and Chute castrated their animals when they were 1 day of age, whereas we castrated ours at 1 month of age. Then, too, Woolley and Chute implanted pellets, each containing 12–15 mg. of the hormone, when the mice were 2 months of age. In our study the hormone was given subcutaneously at a level of 0.1 mg., in peanut oil, daily. The disparity in our results may be explained on such bases as these.

Data were not assembled on the effects of the peanut oil, per se, upon the development of adrenal tumors. Such data ought to be assembled, for it cannot be said with assurance that the carrier itself may not have exerted some influence upon these tumors. These injections did not exert any untoward effects upon the body weights of these mice nor upon their apparent good health. All remained in good condition throughout the test period. Abscesses did not develop as a result of the daily injections, and necrosis of subcutaneous tissues did not occur.

CONCLUSIONS

Our results indicate the tentative conclusion that desoxycorticosterone acetate inhibited the development of adrenal carcinomas in CE mice previously gonadectomized; for when 0.1 mg. of desoxycorticosterone acetate, suspended in peanut oil, was given subcutaneously, daily, to gonadectomized female CE mice for a period of months, the production of adrenal cortical carcinomas was inhibited. In one mouse, which was given the hormone for 6 months, signs of an early carcinoma developed, whereas in all the fifteen untreated controls, carcinomas developed within comparable periods of time. It may well be that the hormone delayed the development of the tumors rather than inhibited them permanently.

Nodular hyperplasia of the adrenal cortex, however, did develop in five gonadectomized animals

of the seven given desoxycorticosterone acetate, but there was no histologic evidence anywhere that either estrogenic or androgenic hormones had been produced. It may well be that desoxycorticosterone acetate may inhibit the elaboration of these steroids or that the degree of nodular hyperplasia in these animals was insufficient to permit an adequate elaboration of them.

Adrenal carcinoma developed in one of twelve gonadectomized male CE mice, an incidence very much lower than anticipated. The adrenals in nine of the twelve showed a nodular hyperplasia, and in one of the twelve mice "Type B" cells were identified in both adrenals. Since susceptible tissues were not stimulated, it was considered unlikely that androgen production had occurred.

The administration of desoxycorticosterone acetate to gonadectomized CE male mice restricted all carcinoma development, although nodular hyperplasia and "Type B" cells developed in some of the animals; so that it would appear that the hormone was not significantly effective when given to castrate male mice of the CE strain.

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Fig. 1.—Bilateral adrenal carcinomas in a CE female mouse killed 8½ months after gonadectomy. Right adrenal weighed 327 mg.; left adrenal weighed 14.0 mg.


METRIC 1
2

Fig. 2.—"Carcinoma *in situ*" occurring in a region of hyperplasia in an adrenal cortex of a gonadectomized female CE mouse (×440).

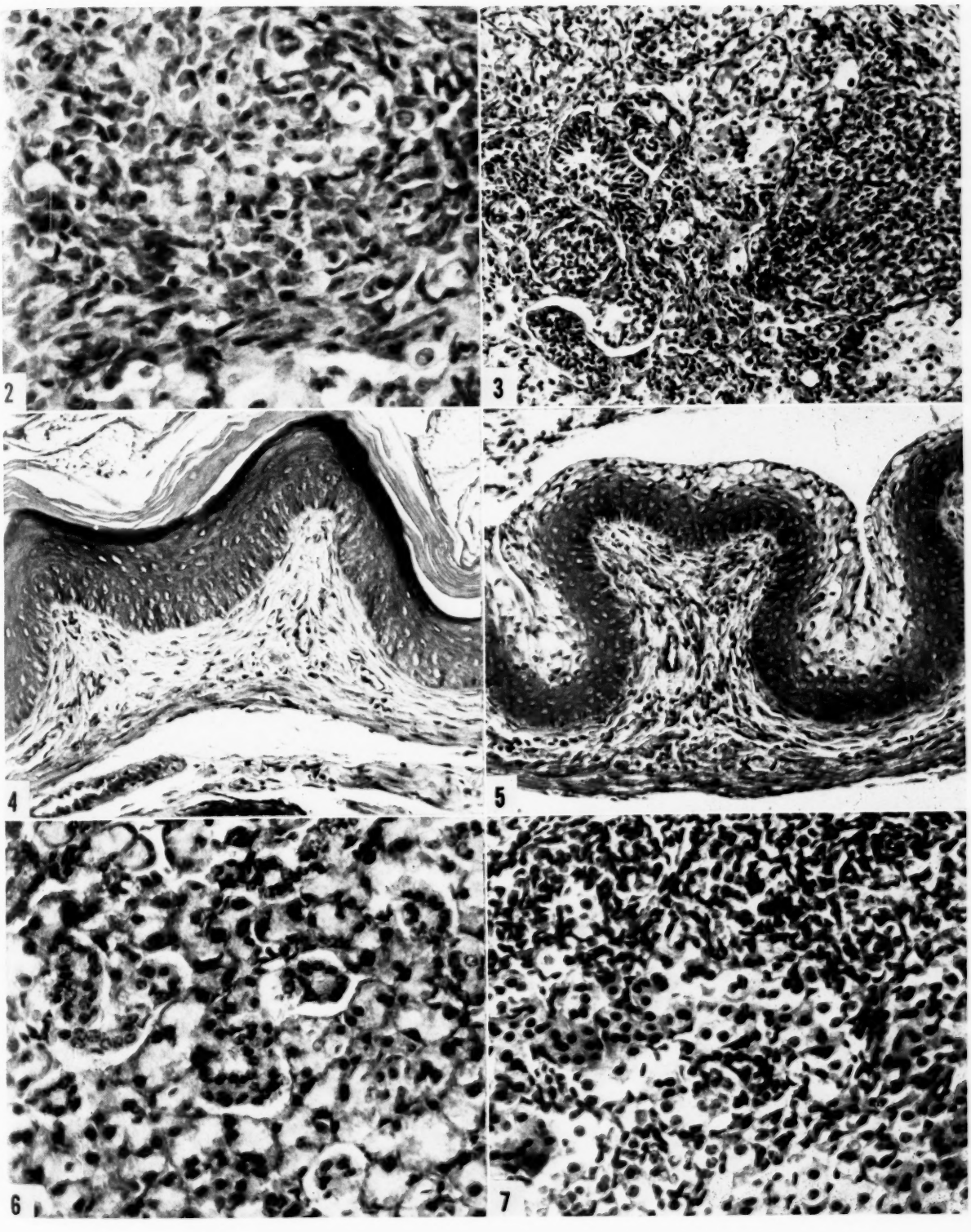
Fig. 3.—Invasive carcinoma of the left adrenal cortex of a gonadectomized female CE mouse (×170).

Fig. 4.—Cornified vaginal epithelium of a gonadectomized female CE mouse (×180).

Fig. 5.—Vaginal epithelium, resembling that characteristic of diestrus, in a gonadectomized CE mouse (×180).

Fig. 6.—Submaxillary gland of gonadectomized CE female mouse with adrenal tumor, showing female type of intralobular ducts wherein nuclei are centrally located in the epithelial cells (×220).

Fig. 7.—Nodular hyperplasia in the adrenal cortex of a gonadectomized CE mouse given 0.1 mg. of desoxycorticosterone acetate daily for 6 months (×330).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Delay of Methylcholanthrene Skin Carcinogenesis in Mice by 1,2,5,6-Dibenzofluorene*†

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This investigation was undertaken to explore the anti-carcinogenic activity of certain organic compounds, some of which are closely related to the carcinogenic hydrocarbons.

Lacassagne *et al.* (2, 3) have reported that the addition of certain weak carcinogens to stronger ones will inhibit the carcinogenic activity of the latter when applied to the skin of mice. They studied the effects of 1,2,5,6-dibenzofluorene and of chrysene when applied with methylcholanthrene, and of 1,2,5,6-dibenzacridine when applied with 1,2,5,6-dibenzanthracene. These workers noted a delay in the onset of the tumors as compared with methylcholanthrene and 1,2,5,6-dibenzanthracene controls. Several methylbenzacridines were found to have no effect on carcinogenesis by 1,2,5,6-dibenzanthracene. They suggested that both the weak and strong carcinogens have an affinity for the same substrate in the cell by virtue of their similar chemical configurations. The weak carcinogen might hinder the fixation of the potent one by the substrate and so delay the onset of tumors.

As a preliminary step in the present investigation, some of the experiments of these workers with 1,2,5,6-dibenzofluorene and chrysene were repeated. In addition, fluorene and 1,2,7,8-dibenzofluorene were tested for possible delaying effects on methylcholanthrene carcinogenesis.

MATERIALS AND METHODS

Twelve-week-old mice of CF1 strain obtained from Carworth Farms, Inc., were used. They were given Rockland Mouse Diet (Arcady Farms Milling Co.) and water *ad libitum*. Each experimental group contained equal numbers of males and females. Acetone was employed as the solvent for all chemicals. One drop (0.02 ml.) of the test solution was applied twice a week to the interscapular region, which was kept free of hair with scissors. Painting was continued for 31 weeks, and charts were kept of all tumors induced. The mice were examined at autopsy, and relevant material was examined histologically.

RESULTS

The effect of 1,2,5,6-dibenzofluorene and of chrysene on methylcholanthrene carcinogenesis.— Three of the first five experiments (Table 1) were carried out as controls. In Experiment 1 the mice were painted with a 0.15 per cent solution of methylcholanthrene, in Exp. 2 with a 0.30 per cent solution of 1,2,5,6-dibenzofluorene, and in Exp. 4 with a 0.20 per cent solution of chrysene. The remaining two experiments were done to test the effects of mixtures. In Exp. 3 a solution containing 0.15 per cent of 1,2,5,6-dibenzofluorene and 0.15 per cent of methylcholanthrene was used, and in Exp. 5 a similar mixture of chrysene and methylcholanthrene was used.

At the end of 31 weeks the mean latent period of tumor induction for each group of animals was calculated for comparison. Mice painted with methylcholanthrene alone had a latent period of 14.1 ± 0.5 weeks, and those with the mixture of 1,2,5,6-dibenzofluorene and methylcholanthrene, 21.3 ± 1.0 weeks. Thus, the addition of the 1,2,5,6-dibenzofluorene caused a delay in tumor induction of 7.1 ± 1.1 weeks.[1] The addition of chrysene to methylcholanthrene had no delaying action.

Five months after starting the first experiments, a second series was begun. Experiments 8

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[1] The difference between the two mean latent periods is shown with the standard error of the difference of the means.

and 9, with a 0.15 per cent solution of methylcholanthrene and a 0.30 per cent solution of 1,2,5,6-dibenzofluorene, respectively, were controls. In Exp. 10 a solution containing 0.15 per cent of 1,2,5,6,-dibenzofluorene and 0.15 per cent of methylcholanthrene was used, and in Exp. 11 The mice in Exp. 6 were given a preliminary treatment by being painted with a 0.30 per cent solution of 1,2,5,6-dibenzofluorene twice a week for 6 weeks. This was followed by the use of the mixture of 1,2,5,6-dibenzofluorene and methylcholanthrene for 25 weeks. The mean latent period of

TABLE 1

LATENT PERIOD AND INCIDENCE OF TUMORS IN MICE PAINTED WITH HYDROCARBONS

Exp. no.	Hydrocarbons*	No. of mice	Survivors at time of first tumor	Number of mice with first tumor and time in weeks																							Total tumor-bearing mice	Tumor-free survivors at 31 weeks	Mean latent period and standard error in weeks
				8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31			
1	0.15 per cent methylcholanthrene control	28	28		1		2	1	6	5	3	2	1	1			1											23	0	14.1±0.5
2	0.30 per cent 1,2,5,6-dibenzofluorene control	20	15														1					1	1	1			1	5	10	26.6±1.6
3	0.15 per cent 1,2,5,6-dibenzofluorene 0.15 per cent methylcholanthrene mixture	20	20							1	2		1	1	1	1	3	3	2			2	1			1		19	0	21.2±1.0
4	0.20 per cent chrysene control	20	16																								1	1	14	31.0±0.6
5	0.15 per cent chrysene 0.15 per cent methylcholanthrene mixture	20	20			2	2	1	3	1	5		2	1														17	0	13.8±0.6
6	0.30 per cent 1,2,5,6-dibenzofluorene for 6 weeks followed by mixture Exp. 3	20	18															3	3	1		2		1	1			11	2	18.4±0.8†
7	0.20 per cent chrysene for 6 weeks followed by mixture Exp. 5	20	19					1				1	1		4	4	4	2	2									19	0	13.7±0.6†
8	0.15 per cent methylcholanthrene control	20	20		2	1	2	1	2	1	2	2	2	3						1								19	0	14.5±0.9
9	0.30 per cent 1,2,5,6-dibenzofluorene control	20	18								2	1					1		2		1	1				1		9	6	21.6±1.8
10	0.15 per cent 1,2,5,6-dibenzofluorene 0.15 per cent methylcholanthrene mixture	20	20						2			2	2	3	1		1	1	1		3					1		17	3	19.8±1.0
11	0.30 per cent 1,2,5,6-dibenzofluorene 0.15 per cent methylcholanthrene mixture	20	20						1			1	4			1	3			2	1					2		15	1	20.9±0.9
12	0.20 per cent 1,2,7,8-dibenzofluorene control	20																										none	19	
13	0.20 per cent 1,2,7,8-dibenzofluorene 0.15 per cent methylcholanthrene mixture	20	20			1			4	3	2	1	2	3			2												18	2	14.7±0.7
14	0.30 per cent fluorene control	20																										none	16	
15	0.15 per cent fluorene 0.15 per cent methylcholanthrene mixture	20	17		1	3	1	2	4	1	1	1	1	1														16	1	11.9±0.7

* Percentage composition of solutions expressed as weight volume per cent.

† Mean latent period calculated from the day that painting with the mixture was begun.

the concentration of the former was increased to 0.30 per cent. The delays in tumor induction, as measured by the differences between the mean latent periods for the mixtures and that for the carcinogen alone, were 5.3 ± 1.4 and 6.4 ± 1.3 weeks.[1] Thus, the results of Exps. 1 and 3 were confirmed by Exps. 8, 10, and 11.

The effect of preliminary painting with 1,2,5,6-dibenzofluorene and with chrysene.—Experiments 6 and 7 were also of a type described by Lacassagne. tumor induction was 18.4 ± 0.8 weeks, when calculated from the day that painting with the mixture was begun. Since the latent period for control Exp. 1 was 14.1 ± 0.5 weeks, this represented a delay of 4.3 ± 0.9 weeks.[1]

In Exp. 7 the 6 weeks' preliminary treatment with chrysene was followed by painting with a mixture of chrysene and methylcholanthrene for 25 weeks. The mean latent period, as calculated from the beginning of treatment with the mixture, was

13.7 ± 0.6 weeks. This latent period was not significantly different from that of 14.1 ± 0.5 weeks for the methylcholanthrene control.

The effect of 1,2,7,8-dibenzofluorene and of fluorene on methylcholanthrene carcinogenesis.—Experiments 13 and 15 show that neither the closely related 1,2,7,8-dibenzofluorene nor the parent compound, fluorene, has any delaying effect on methylcholanthrene carcinogenesis. The mean latent period for the mixture of fluorene and methylcholanthrene was 11.9 ± 0.7 weeks, which suggested that the fluorene may have enhanced the action of methylcholanthrene (mean latent period, 14.5 ± 0.9 weeks).

Results of autopsy.—Autopsies were performed up to 48 weeks after the beginning of the experiments, although painting of the skin was stopped at the end of the 31st week. There were no significant differences in weight between the mice receiving the carcinogen alone and those receiving the mixture. Table 2 shows the distribution of squamous-cell tumors encountered histologically. Where multiple tumors were present in an animal, only that tumor showing the greatest progression is recorded. The table also shows the incidence of sarcomas occurring either in association with a squamous-cell tumor (carcinosarcoma) or independently in the painted area.

DISCUSSION

The experiments indicated that the addition of 1,2,5,6-dibenzofluorene to methylcholanthrene in acetone caused a statistically significant delay[2] in the time required for the development of skin tumors in CF1 mice. The effect of 1,2,5,6-dibenzofluorene was the same whether its concentration was equal to or double that of the methylcholanthrene. These results confirm those of Lacassagne *et al.* (2, 3) in respect to the delaying action of 1,2,5,6-dibenzofluorene on methylcholanthrene skin carcinogenesis in mice.

Chrysene, when used with methylcholanthrene, had no delaying action. This is contrary to the findings of the French workers.

A preliminary treatment with 1,2,5,6-dibenzofluorene for 6 weeks, followed by painting with the mixture of this chemical and methylcholanthrene, had been used in an attempt to obtain a greater delay than that provided by the mixture alone (3). In the present repetition of this experiment, the preliminary treatment failed to prolong the latent period beyond that obtained by applying the mixture alone. In the similar experiment with chrysene there was no delaying effect whatever.

Anti-carcinogenic activity of the type demonstrated by 1,2,5,6-dibenzofluorene is not a general property of noncarcinogenic or weakly carcinogenic hydrocarbons, since it is not possessed by chrysene, fluorene, or 1,2,7,8-dibenzofluorene. The anti-carcinogenic activity has affected only the latent period of tumor induction and has not affected significantly the total tumor incidence. The mechanism of the delaying action of 1,2,5,6-dibenzofluorene remains unknown.

TABLE 2

HISTOLOGICAL ANALYSIS OF TUMORS

Exp. no.	No. mice examined	No. mice without squamous-cell tumor	Distribution of tumor types			
			Papilloma*	Carcinoma†		
				I	II	III
1	21	1	0	1	19(7)‡	0
2	10	4	0	1	5(1)	0
3	16	2(1)‡	0	1(1)	12(2)	1
4	13	12	0	0	1	0
5	16	0	0	1	13(3)	2
6	17	5	0	1	9	2
7	17	0	0	1	11	5
8	15	0	1	2	9(1)	3
9	14	5	0	0	7(2)	2
10	17	3(1)	0	4	8	2
11	17	4(1)	0	2	11(3)	0
12	18	18	0	0	0	0
13	17	1(1)	0	3	11	2
14	15	15	0	0	0	0
15	16	0	0	0	13(1)	3

* Squamous cell tumor, well differentiated, noninvasive.

† Squamous cell tumor, classified as follows: I. Focal pronounced cellular variation, little or no invasion. II. Cellular variation and invasion. III. Cellular variation, invasion, and metastasis.

‡ Numbers in parentheses indicate animals in which the dermal or stromal connective tissue was polymorphic and invasive.

SUMMARY

1. The addition of 1,2,5,6-dibenzofluorene to methylcholanthrene delays skin carcinogenesis in mice.

2. Chrysene, fluorene, and 1,2,7,8-dibenzofluorene have no delaying effect on methylcholanthrene skin carcinogenesis in mice.

ACKNOWLEDGMENT

The authors wish to thank Mr. Robert Feldman for his technical assistance.

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[2] Test and control groups were compared statistically on the basis of the mean latent period of tumor induction by calculating the value of P (1). For each pair of groups, Exps. 3 and 1, 6 and 1, 10 and 8, and 11 and 8, the value of P was less than 0.01.

Increased Weight and Mitotic Activity in the Liver of Tumor-bearing Rats and Mice

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E. Yeakel (4) reported on changes in liver weight as compared to body weight in Wistar albino rats bearing induced or transplanted tumors. He concluded that the liver weight of the tumor-bearing host is increased yet does not show any correlation either to the body weight or to the weight of the tumor.

Autopsies carried out in this laboratory on rats and mice bearing spontaneous or grafted tumors showed a relationship between the weight of the tumor and that of the liver/body ratio. Yeakel suggests that the increased size of the liver of the tumor-bearing animal is related to the increased anabolism of proteins induced by the tumor. Histological examinations of the livers of the tumor-bearing hosts offer an alternative explanation.

PROCEDURE

Four kinds of malignant tumors were examined for their effect on the liver. Experiments have been carried out on rats and mice of both sexes and of various weights, bearing one of the following tumors: reticuloendothelioma of the rat; fibrosarcoma of the albino mouse; Meyer tumor (a mammary tumor of Brazilian origin) of the albino mouse; spontaneous and transplanted mammary carcinoma of the C3H strain mouse.

The results were calculated on the basis of the ratio liver/body, i.e., the gram value of the liver related to 1 gm. of body of the exsanguinated animal deprived of its tumor. Tumors were weighed separately. Tumors were used when no necrosis was found upon dissection. In rats, even large tumors were not necrotic, but in mice necrosis of the tumor was more frequent, and all open necrotic tumors were discarded. All animals had been kept on a stock diet consisting of 75 per cent maize flour, 8 per cent casein, 6 per cent meat powder, 3 per cent lard, 6 per cent peanut oil, 1 per cent cod liver oil, and 0.3 per cent salt mixture.

To determine the mitotic activity of the liver in tumor-bearing animals, histological sections of liver and in some instances of kidneys were prepared. They were compared to sections from healthy animals. The mitotic activity was expressed by the mitotic index, i.e., the number of mitoses present in 100 microscopic fields at a magnification of 950. At least 200 fields were counted.

Unilateral nephrectomy was carried out on some C3H strain mice. The livers and kidneys of these animals had been examined after an interval of 14 days.

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RESULTS

Results obtained are presented in Tables 1 and 2 and Figure 1. Data for rats indicate that the numerical value of the liver/body ratio shows a fairly constant value. In the case of tumor-bearing rats, the ratio liver/body shows a well defined increase if the tumor reaches an optimal weight. Mitoses were found in every case.

The numerical value of the liver/body ratio for mice shows a greater deviation than that for rats. Nevertheless, the numerical value increases significantly in the presence of tumor. Mitoses are not always detectable in the liver of tumor-bearing mice.

Unilateral nephrectomy of tumor-bearing C3H strain mice led to a highly increased mitotic activity, with abnormalities in mitotic figures in a large portion of the livers examined.

No mitotic activity was observed in the livers of control animals, nor could mitoses be detected in the kidneys of either control or tumor-bearing animals.

DISCUSSION

The results presented are in agreement with those obtained by Yeakel (4) As these examinations included mice bearing different kinds of tumors, as well as rats, the results may be interpreted from a broader point of view. A strict numerical correlation of tumor weight related to the ratio liver/body weight should not be emphasized. In general, it seems that the liver enlargement of the host animal is already detectable when the size of the tumor is still relatively small,

and it reaches its greatest extent with the heaviest tumor. It is probable that the extent of the liver enlargement depends on the ability of the liver to respond to stimuli given by the tumor.

Varied factors may be involved in the enlargement of the liver, and up to this time only a few are known. The increase in the percentage of water, as found by McEwen and Haven (3), although only in limited amounts, seems to be important. It suggests the possibility of systemic changes in the cytoplasm. The suggestion of Yeakel that the increase in liver size is induced by hypertrophy leading to an increased protein anabolism is still to be proved.

Histological investigations indicate that the increased weight of the liver may be explained in part by a mitotic activity induced by the presence of the tumor. The liver of tumor-bearing rats reacted uniformly: mitoses were increased when the tumor was still small (Table 1, Nos. 1, 2). On

TABLE 1

THE RELATION OF BODY WEIGHT TO THE SIZE AND MITOTIC RATE OF THE LIVER
(Rats of Both Sexes, Bearing Transplanted Reticuloendotheliomas)

No.* of Rat	Tumor weight, (gm.)	Ratio L/B†	Mitotic index (per cent)
1	0.12	0.031	0.5
2	6	0.035	3.0
3	17	0.057	8.0
4	27	0.044	1.5
5	39	0.041	4.5
6	43	0.047	11.5
7	46	0.067	4.0
8	47	0.040	3.5
9	56	0.044	2.5
10	62	0.046	5.0
11	74	0.052	11.5
12	87	0.054	10.0
13	92	0.072	6.5
14	93	0.064	3.5
Controls 8 animals	0	0.031±0.0023	0

* Number represents single animal.
† L/B = gm liver wt/gm body wt.

the other hand, there was no relation between the degree of the mitotic activity and the size of the liver.

Mitoses in the liver of mice bearing spontaneous or grafted tumors could not be observed in each case. It appears, however, that the liver enlargement in these animals may also be explained by the increase in mitoses. Kelly and Jones (2) showed recently that the desoxypentosenucleic acid is increased in the livers and kidneys of tumor-bearing animals. They suggest that this increase may be responsible for the greater mitotic activity in those organs.

It has been supposed that the agent related to mitotic activity is a product or constituent of the malignant cell which is carried via the bloodstream to the liver. A portion of it is retained by the liver and induces mitoses in that organ, while the remainder is excreted by the kidneys. Thus, it appears that the simplest way to concentrate it in the liver is to delay its urinary excretion by the

TABLE 2

THE RELATION OF BODY WEIGHT TO THE SIZE AND MITOTIC RATE OF THE LIVER
(Mice of Both Sexes, Bearing Transplanted and Spontaneous Tumors)

No.	Kind of tumor	Tumor weight (gm.)	Ratio L/B	Mitotic index (per cent)
ALBINO MICE				
1	Meyer tumor	3	0.057	6.0
2	"	3	0.057	4.5
3	"	4	0.050	0
4	"	5	0.062	12.0
5	"	10	0.072	7.0
6	Fibrosarcoma	2	0.057	1.5
7	"	2	0.060	9.0
8	"	5	0.067	3.0
Controls 8 animals		0	0.038±0.0045	0
C3H STRAIN MICE				
1	Mammary tumor	0.2	0.042	0
2	"	2	0.057	2.0
3	"	3	0.054	2.0
4	"	6	0.093	0.5
5	"	3	0.044	4.0
6	"	5	0.070	0
7*	"	3	0.060	0.5
8*	"	3	0.052	1.0
9*	"	2	0.057	6.0
Controls 8 animals		0	0.037±0.0043	0
C3H STRAIN MICE WITH UNILATERAL NEPHRECTOMY				
1	Mammary tumor	2	0.060	23.5
2	"	4	0.066	20.0
3	"	8	0.088	7.0
4	"	8	0.085	46.0
5	"	2	0.067	7.0
6	"	2	0.062	11.0
7	"	2	0.069	1.5
Control 1	0	0	0.042	0
Control 2	0	0	0.047	0

* Spontaneous tumors.

removal of one kidney. This is difficult to prove, however. In any case, the significant rise in the number of dividing cells and the abundance of atypical mitoses in the liver of nephrectomized tumor-bearing C3H strain mice may support the supposition that in the liver of tumor-bearing mice the biological equilibrium is disturbed.

The results recently obtained by Bucher and associates (1) on the regeneration of the liver in parabiotic rats also favors the hypothesis of a blood-borne factor which may be involved in liver

mitoses. The question of the number of mitoses in normal liver of rodents is best described by Bucher, who states: "Mitoses are extremely rare in the liver of normal adult rats—approximately one in 34,000 cells." That would mean that she counted, at the most, 3 mitoses within 100,000 cells. Thus, a very low probability existed that the present authors would observe mitoses in microscopic fields containing approximately 42–45 cells (Mag. × 950).

Neither mitoses nor other kinds of abnormalities could be detected in the liver of nephrectomized, healthy, control animals.

SUMMARY

1. It was determined at autopsy of rats and mice bearing malignant tumors that a more or less close relation existed between the weight of the tumor and that of the liver, calculated on the basis of the ratio liver/body. Four kinds of malignant tumors were examined for their effect on the liver.

2. The increase in the weight of the liver of the tumor-bearing animal seems to be partly a consequence of mitotic activity induced by the presence of the tumor.

3. Unilateral nephrectomy in tumor-bearing C3H strain mice evoked an increase in mitoses. Mitoses appear throughout the whole liver, showing a great variety of abnormalities.

4. Control animals showed no trace of mitotic figures.

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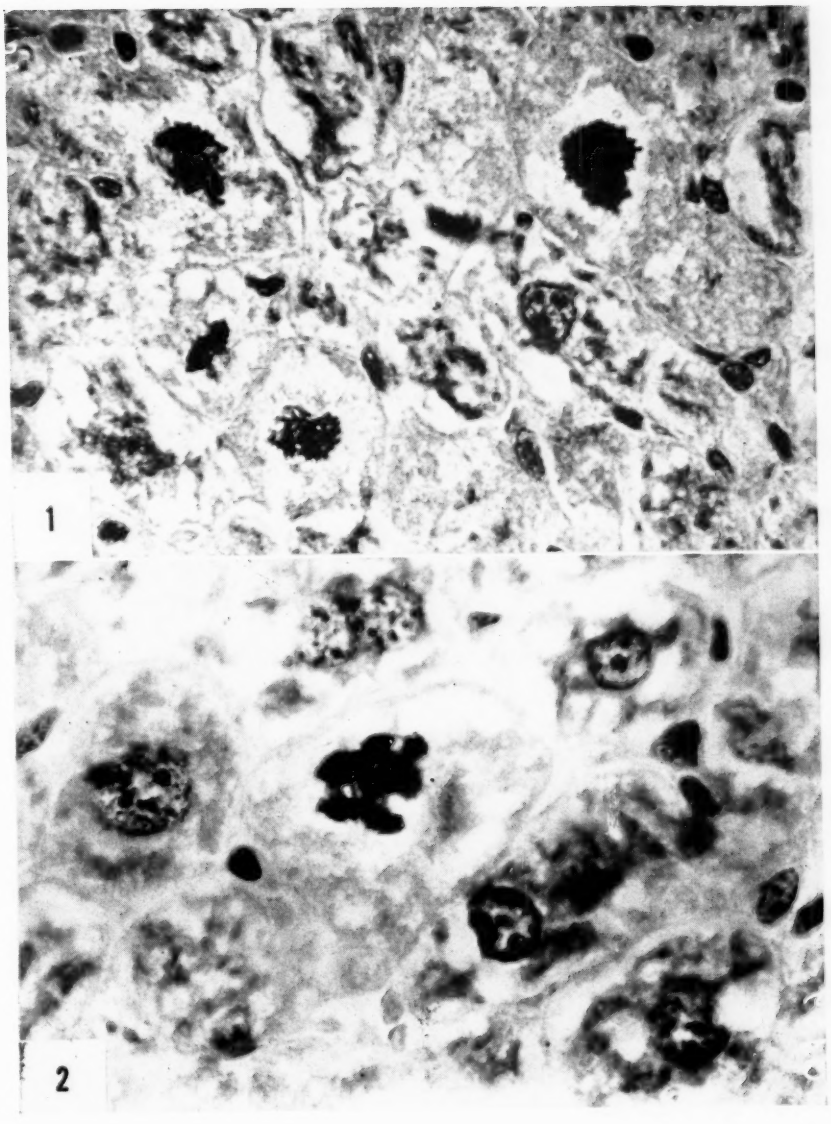
Fig. 1.—Mitotic cells in the liver of tumor-bearing C3H strain mouse after unilateral nephrectomy. Experiment No. 10. Hematoxylin and eosin, ×1,200.

Fig. 2.—Atypical mitosis in the liver of the same mouse. Hematoxylin and eosin, ×1,200.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Further Studies on the Recovery of 2-Acetylaminofluorene from Rats Following Oral Administration

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Morris and Westfall (5) found, after a single feeding of 2-acetylaminofluorene (AAF) to rats, that tissue concentrations of the carcinogen were greatly decreased, or in some cases no longer detectable, well before the sixteenth hour, while considerable amounts of the material still remained in the stomach contents. They concluded that the AAF may have been rapidly "inactivated." It was also suggested by them that part of the carcinogen may have entered into chemical combination with some tissue constituent, resulting in an acetone-insoluble conjugate. Since their procedure recovered only acetone-extractable AAF or aminofluorene (AF), such a conjugate would not have been detected. The present investigation was undertaken with the purpose of exploring the second possibility in an effort to find the large percentage of administered diazotizable material that could not be accounted for in the acetone extracts of the stomach, tissues, and excreta of the rat 5–24 hours after gastric instillation with AAF.

The fact that 7-hydroxy-2-acetylaminofluorene has been isolated from the urine of rats following oral administration of AAF (1) suggested the likelihood of an ester linkage with fatty acid if a hydroxy derivative of the carcinogen was present in the tissues. Suitable extracts of the tissues of rats that had received AAF either by stomach tube or by feeding were therefore saponified before analyzing for diazotizable material.

The expected ease of deacetylation, proof of which has recently been found for AAF by Morris, Weisburger, and Weisburger (4), made feasible the assumption that some AF was produced in the body following administration of AAF and that the amino group might enter into peptide linkage with free carboxyl groups of tissue proteins. Consequently, protein fractions of groups of tissues or of the whole animal, after administration of AAF, were hydrolyzed with HCl, KOH, or trypsin, preceding ether extraction and diazotization of the ether residues.

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METHODS

Method of Analysis for Aminofluorene

A slightly modified adaptation of the diazotization method of Westfall and Morris (11) was used. The modification is described as follows: three tubes containing the dried residue of an acetone or ether extract of the test material in a solution of 1 volume of glacial acetic acid, 0.5 volume of concentrated HCl, and 4 volumes of distilled water were heated for 1 hour in a boiling water bath. The tubes were then immersed in ice water, and the volumes of the solutions were made up to 3.0 ml. by the addition dropwise of a mixture containing the same proportions of the acids and water. To one of the three tubes was added 1 ml. of distilled water, and to two of the tubes was added 1 ml. of 0.03 M aqueous sodium nitrite. Each tube was shaken after the addition of the water or of the sodium nitrite and was quickly returned to the ice bath. After 1 minute (timing is important), 2 ml. of the contents of each tube (total volume was then 4 ml.) was pipetted with shaking into test tubes (fitted with glass stoppers) containing 5 ml. of the ammoniacal R salt solution (0.03 M R salt in 5 M NH_4OH). To each tube was added 2.5 ml. of 92 per cent acetone. The tubes were stoppered and the contents shaken. The red color, which is formed by coupling of the AF through the diazonium compound with R salt, was measured in a Coleman Universal spectrophotometer, Model 11, using a wave length of 525 mμ.[1] The tube that received water instead of sodium nitrite was used as the blank for each determination. Although the method of analysis, with the procedure of heating in a boiling water bath for 1 hour in 1 N HCl, measures AF, the material administered was AAF, and that recovered was calculated as AAF.

Since the diazotization test was found to be negative with tissues of normal rats and positive with tissues of rats following administration of

[1] This wave length corresponds to the maximal spectral absorption for the 2-fluorenyl-azo-1-naphthol-3,6-disulfonic acid sodium salt as demonstrated by the spectral absorption curve published by Westfall and Morris (11).

AAF or AF, the assumption is made that the chromogenic material measured was one of these compounds or closely related diazotizable derivatives and that it came from the carcinogen. Throughout the paper, therefore, the terms AF and AAF, or "specific" diazotizable material have been used to designate the organic solvent-soluble material which reacted with R salt to give a chromogen having the same absorption spectrum as that of 2-fluorenyl-azo-1-naphthol-3,6-disulfonic acid sodium salt.

Sensitivity of method.—The results of pilot recovery experiments of small amounts of AAF added to tissues before and after homogenization in acetone confirmed the findings of Westfall and Morris (11), who noted excellent agreement between replicate estimations but an absolute, constant error below 10 μg.—the source of which appeared to be in the preparation of the extracts. Preliminary recovery experiments, however, with AAF added to tissues and carried through the various procedures necessary for the hydrolysis of tissue constituents, demonstrated that recovery was not quantitative, especially with the hydrolysis of protein material in which the losses varied, with different tissues, from 35 to 75 per cent. This observation led us to set up, for each analysis, control flasks containing equal amounts of the particular tissue being analyzed but with AAF added. The routine addition of 300 μg. of AAF to the control tissue was chosen, because that amount provided sufficient material for duplicates and blanks, even when partial disappearance of the added AAF occurred. When most of the AAF was recovered, however, it was necessary to make at least a 1:5 dilution of the final extract for analysis. Since the search was for unaccounted-for quantities of AAF ranging from 5 to more than 20 mg., the use of 300 μg. in the control flasks is believed to be justifiable, especially since the individual analyses represented large fractions of the tissues of the entire rat. Moreover, the experimental tissues yielded completely negative results following the hydrolytic procedures. The results are therefore qualitative and significant.

Treatment of Rats

Male and female rats of the Buffalo strain were used. They were bred at the National Cancer Institute and were maintained, up to the experimental period, on N.C.I. pellets (3). Two types of experiments were set up: one in which a large single dose of AAF in propylene glycol was administered by stomach tube to rats that had been on fast, and the second type in which AAF was incorporated in the food for several weeks or months before the test period. Two different diets[2] were used for the feeding of AAF, both of which have been found in this laboratory to be carcinogenic to the Buffalo strain of rats. One of the diets was a semi-synthetic type, containing, in addition to the carbohydrate and vitamins, 12 per cent commercial casein and 21 per cent lard in which was incorporated AAF to give 1.2 mg/gm of food. The second diet was prepared from natural foodstuffs and contained approximately 22 per cent protein from several sources and 10 per cent fat, the fat again having been used to dissolve AAF, this time to give 0.25 mg/gm of diet.

The test rats were placed in metabolism cages for fasting periods of 16–23 hours (and of 5 hours for 2 rats) following the administration, or the last day of ingestion, of the carcinogen. Feces and urine were thus available for analysis. Blood was removed by cardiac puncture with the rat under ether anesthesia. The animal was then killed, and the tissues were removed and weighed. They were homogenized in a Potter-Elvehjem homogenizer, or, in the case of the whole rat, the animal was skinned, the organs were then homogenized in a Potter-Elvehjem homogenizer and the muscle, skin, and skeleton in a Waring Blendor following repeated freezing and mincing in a tissue grinder. The extraction solvent depended upon the particular procedure which was being followed, as indicated below.

Preparation of Tissues for Analysis

Acetone extraction of tissues.—Some homogenates were made in acetone according to the procedure followed by Westfall (10).

Ether extraction of materials.—Aqueous solutions such as blood, urine, hydrolysates of tissues and (or) saponified extracts were extracted with ether instead of acetone, acid extracts being neutralized before ether extraction. After evaporation of the solvent, the ether residue was analyzed.

Isolation of soluble proteins in acetate buffer.—The tissues were homogenized in water and extracted in ice-cold 0.5 M sodium acetate buffer of pH 5 following, with slight modification, the method used by Miller and Miller (2). The dried crude protein material was hydrolyzed as described below, and the hydrolysates were analyzed for the "specific" diazotizable material. Alcohol extracts and washes were evaporated at room temperature (or at reduced pressure), and the residues were saponified and then treated according to the procedure given under "Saponification."

Treatment of tissue residues.—The residues of

[2] These diets will be described in detail in subsequent publications.

tissues remaining after extraction with acetate buffer were extracted with acetone. The acetone was evaporated, and the acetone-soluble residues were analyzed. Samples of the tissues remaining (final residues) after extraction with both acetate buffer and acetone were hydrolyzed with potassium hydroxide in the autoclave and with trypsin, by the same procedures as those used for the isolated, coagulated, acetate-extracted, protein material. In two experiments portions of the final residues were also hydrolyzed with HCl.

Saponification

To test for the presence of AAF conjugated with fatty acids, residues of acetone, ether, and alcohol extracts, after evaporation of the solvents, were subjected to saponification. After cooling, the saponified material was extracted with ether in separatory funnels, the ether extracts were washed with water until free of alkali, the solvent evaporated, and the ether residues analyzed as described. Recovery experiments with 300 μg. of AAF added to the same weights of tissue and carried through the same extraction, evaporation, saponification, etc., procedures demonstrated that 95–100 per cent of such added material could be recovered.

Hydrolysis of Protein Material

Hydrolysis with KOH.—Since AAF could not be detected in the hydrolysates after refluxing protein material containing 300 μg. of AAF with 4.5 N alcoholic KOH for 20 hours, according to the method of Miller and Miller (2), the hydrolysis was accomplished by autoclaving at 20 lb. pressure in the presence of KOH.

A study of the influence of alkali concentration and autoclaving time upon the recovery of AAF and upon the conversion of protein nitrogen to nonprotein nitrogen led to the use of a concentration of 1.0 N aqueous KOH for a period of 3 hours at 20 lb. pressure. In the presence of 0.2–1.0 gm. of proteins and of 5–10 gm. of tissue residues, the recovery of 300 μg. of AAF added to the control flasks varied from as little as 25 per cent, with final residues of muscle, to 66 per cent with final residues of miscellaneous organs and with acetate buffer-soluble proteins. Under these conditions, control samples of 300 μg. of AAF, in the absence of rat tissues, were recovered to the extent of 75 per cent.

Hydrolysis with HCl.—The acetate buffer-soluble proteins and the acetate acetone-extracted residues (final residues) of two rats that had received 20.3 and 19.5 mg. of AAF in propylene glycol by stomach tube 23 hours before killing were hydrolyzed by refluxing for 24 hours in 20 volumes of 20 per cent HCl. The hydrolysates, after concentration *in vacuo*, were made slightly alkaline with NaOH and were extracted with ether in a separatory funnel. The recovery of 300 μg. of AAF added to isolated protein material or to the protein-containing tissue residues was approximately 35 per cent when hydrolysis was carried out in this manner. Acetylaminofluorene, refluxed in 20 per cent HCl for 24 hours in the absence of protein material, then made alkaline and extracted as described, resulted in a recovery of 65 per cent of that added, thus representing considerably less loss than when protein was present to be hydrolyzed.

Hydrolysis with trypsin.—Because of the failure to recover AAF quantitatively from either acid or alkaline digests of protein material, tryptic digestion was tried, with 1–10 gm. of the protein material, and with digestion for 7 days at 40° C. with "Difco" standardized (1:250) trypsin at pH 8.3 ± 0.3. Toluene and chloroform were used as preservatives. The digests and preservatives were then extracted with ether in a separatory funnel, and, after volatilization of the ether, the residues were analyzed by the usual procedure. In the presence of large amounts of fatty material the ether residues were saponified preceding analysis.

Samples were removed before and after digestion for determination of total and nonprotein nitrogen. With some of the insoluble tissue residues, total nitrogen, before digestion, had to be determined with dry samples, since accurate sampling of the suspensions was impossible. The recovery with 300 μg. of AAF added to the control flasks was between 84 and 92 per cent for all digests except those of the carcass and skin with hair. With these latter tissues there was present undigested residue, and the recovery of added AAF varied from 40 to 60 per cent.

Treatment of Urine for Detection of the Glucuronide of Hydroxyaminofluorene

Neish (6) isolated the glucuronide of 2-hydroxyfluorene from rabbits' urine following daily administration of fluorene. In a search for the possible presence of the glucuronide of hydroxyaminofluorene, the urine, after ether extraction for the determination of unconjugated AF and AAF, was made approximately 0.2 N with HCl or with H_2SO_4 and was refluxed for 3 hours. After hydrolysis the treated urine was neutralized and again extracted with ether. The residue, after volatilization of the solvent, was analyzed. Recovery of the diazotizable material after addition of AAF to urine before refluxing was not quantitative, since not more than 60 per cent of 300 μg. of added AAF could be accounted for.

RESULTS

No diazotizable material giving the color reaction of AF with R salt was liberated from the hydrolysates of lipid or of protein material from rats' tissues following administration of AAF. It is evident, therefore, that the data presented in Tables 1–3 represent the recoveries from acetone extracts of the tissues and excreta of the rats. The last columns of Tables 1 and 2 list the different procedures used for the analyses.

Saponification of the residues from acetone, ether, or alcohol extracts of the tissues and excreta made possible the analysis of material containing large amounts of fat without the loss of AF or AAF as a result of their solubility in fat. Since tissues that contained moderate amounts of lipid material yielded the same amount of AF or AAF with and without saponification, it became apparent that saponification did not liberate significant amounts, if any, of the diazotizable material.

TABLE 1

RECOVERY OF ACETYLAMINOFLUORENE* FROM RATS AFTER A SINGLE ADMINISTRATION BY STOMACH TUBE

No. of rats	Total weight of rats (gm.)	Amount AAF administered (mg.)	Interval following administration of AAF (hours)	Per cent recovered†						Procedures used (No.)‡
				Total	Gastro-intestinal tract	Feces	Urine	Blood	Remainder of rat	
2	398	39.8	23	24.7	22.0	0	2.57	0	0.08	a, i; d, e; m; f, l; h, i
1	251	32.2	16	27.8	24.2	0	0.75	Trace	2.8	a, i; b, i; d, e
2	433	34	5	32.06	30.1		1.16	0.09§	0.71	a, i; f, j, k; h, i, j, k; d, e; m
2	539	55.5	5	65.7	64.2		1.0	0.09§	0.41	a, i; c, i, j, k; d, e; m

* Could also have been aminofluorene or structurally related diazotizable derivatives.

† Per cent recovered, based on amount injected.

‡ Procedures used in analysis of tissues:

a) Acetone extraction of gastrointestinal tract with contents, and separately, of feces.
b) Acetone extraction of liver, miscellaneous organs, muscle, and skeleton-skin-hair of rat, separately.
c) Acetone extraction of tissues of entire rat except gastrointestinal tract.
d) Ether extraction of urine.
e) Ether extraction of blood.
f) Isolation of acetate buffer-soluble protein material of peritoneal organs, and separately, of remainder of rat except gastrointestinal tract.
g) Isolation of acetate buffer-soluble protein material of tissues from the whole rat, except the gastrointestinal tract.
h) Acetone extraction of tissue residues following extraction with acetate buffer, leaving "final residues" of tissues.
i) Saponification of residues from organic solvents.
j) Hydrolysis of protein material and of "final residues" with 1 N KOH in autoclave.
k) Hydrolysis of protein material and of "final residues" with trypsin.
l) Hydrolysis of protein material and of "final residues" with HCl.
m) Acid hydrolysis of aqueous layer of ether-extracted urine.

§ The calculated proportion of that recovered from the total pooled blood from the four rats.

TABLE 2

RECOVERY OF ACETYLAMINOFLUORENE* FROM RATS 17–22 HOURS† AFTER REPEATED INGESTION

No. of rats	Period of feeding (days)	Total amount AAF ingested (mg.)	Calculated average daily intake AAF‡ (mg.)	Per cent recovered§						Procedures No.‖
				Total	Gastro-intestinal tract	Feces	Urine	Blood	Remainder of rat	
6	18	392.4	21.8	0.34	0	0	0.34		0	a; a, i; b; b, i; i; d, e
1	3	12	4.6						0.16	f, j, k; h, i, j, k
2	20		13.46	1.75	0	1.4	0.22	0	0.13	a, i; g; j, k; h, i, j, k; d, e; m
2	33	604	17.2	1.61	0	0.96	0.50	0	0.15	a, i; g; j, k; h, i, j, k; d, e; m
2#	215	1,023	5.6	0.80	0	0	0.80	0	0	a, i; g; j, k; h, i, j, k; d, e; m
1#	222	593	3.1	0	0	0	0	0	0	a, i; c, i, j, k; d, e; m

* This could also have been aminofluorene or structurally related derivatives.

† The rats were kept in metabolism cages during fasting periods.

‡ This represents the calculated average amount of AAF ingested during the 24 hours preceding transfer to metabolism cage.

§ Per cent recovery is based on the calculated amount AAF ingested during last 24 hours preceding fast in metabolism cage.

‖ See footnote ‡ in Table 1 for guide to procedures.

These rats were carrying primary subcutaneous tumors induced by AAF.

Although tryptic digestion failed to yield any diazotizable material giving the specific color reaction, all the protein materials became completely soluble during digestion except the acetate–acetone-extracted and the acetone-extracted residues of skin and skeleton and of whole rats in which there remained some insoluble residues consisting largely of bone and hair. Between 90 and 95 per cent of the total nitrogen of the soluble digests was converted to nonprotein nitrogen during digestion.

One series of tryptic digests was analyzed for amino acid nitrogen by the ninhydrin titration method of Van Slyke, MacFayden, and Hamilton (9). The α-amino acid nitrogen found was 36 per cent of the total nitrogen of a digest of the acetate-extracted proteins, 29.5 per cent in the digest of the acetone-extracted residue, and 18.9 per cent of the total nitrogen of the digest of the acetate–acetone-extracted final residue of whole rats.

While hydrolysis of the acetate-soluble protein material and the protein-containing residues of tissues, by autoclaving in potassium hydroxide and by refluxing in hydrochloric acid solution, resulted in the loss of considerable portions (varying in amounts with different tissues) of added AAF, no trace of AF or AAF was detected in any of the hydrolyzed material from rats to which AAF had been administered. These methods of hydrolysis also effected the solution of the substrates and the conversion of 90–95 per cent of the total nitrogen to nonprotein nitrogen.

Small amounts of some "specific" diazotizable material were recovered from the ether extracts of urine of rats during the collection periods of 16–23 hours following a single oral administration of AAF. Following this ether extraction, the urine that was made acid, refluxed, neutralized, and again extracted with ether yielded an additional small amount of diazotizable material which may have been present in the urine as the glucuronide, since this treatment would be expected to hydrolyze a glucuronide. The ether extracts of urine of normal untreated rats gave negative results in this test before and after acid hydrolysis. If the percentage losses in the experimental urines were proportional to those in which AAF was added to urine before refluxing in acid medium, the amounts of material excreted in this form in the urine would still represent insignificant fractions of the total amount of the AAF administered.

TABLE 3

DISTRIBUTION OF ACETYLAMINOFLUORENE* IN TISSUES OF RAT 16 HOURS AFTER A SINGLE ADMINISTRATION BY STOMACH TUBE†

	AAF RECOVERED			
TISSUE	Total amount (μg.)	Per gm. tissue (μg.)	Per cent of total administered	Per cent of total recovered
Feces‡	0	0	0	0
Urine	244	16.2	0.75	2.70
Gastrointestinal tract	7,800		24.20	86.8
Kidneys and adrenals	16	9.8	0.049	0.17
Lungs	8	6.8	0.024	0.085
Liver	40	5.8	0.12	0.44
Remainder of rat§	869	4.1	2.60	9.60
Blood	0	0	0	0

* Could also be aminofluorene or structurally related derivatives.

† 32.2 mg. of AAF in 3.92 ml. propylene glycol = 12.8 mg. AAF/100 gm body weight.

‡ Only 58 mg. feces were excreted.

§ Remainder of rat consisted of genitals, brain, muscle, skin, subcutaneous fat, hair, and skeleton.

DISCUSSION

Whether or not the failure to account quantitatively for the AAF added to proteins after acid or alkaline hydrolysis is a result of destruction of the carcinogen or of combination of the amino group with metabolites present in the hydrolysate is not known. Since the method used for analysis requires the presence of the nitrogen of the amino group for diazotization, deamination of the carcinogen would result in failure of recovery. Moreover, any linkage of the amino group that could not be broken by hydrolysis would result in failure to detect the material. Neish (7) has shown that AF condenses with pyruvic acid *in vitro* to yield a cinchoninic acid derivative, 6-methyl-2,3-(fluoreno)-2′,1′-pyridine-4-carboxylic acid. Since the nitrogen of this compound is present in the pyridine ring, it would not be diazotizable. Another reaction that would result in the formation of nondiazotizable material has been suggested by the observation of Neish (8) of a reaction *in vitro* between AF and dihydroxymalonic ester to yield ethylfluorene dioxindole carboxylate-2′,3′-indeno-4,5-isatin and the oxime of this product.

The failure to detect any of the administered

AAF in hydrolysates of proteins or of lipids and the small fraction of the total administered AAF accounted for in the acetone extracts of the tissues and excreta by the diazotization method suggest the necessity and rationality of following the metabolic path of this carcinogen through the body by means of tracer atoms. While this work was in progress, Morris and associates (4) studied the distribution of radioactivity in the tissues of the rat with carbon-14 in the 9 position and with carbon-14 in the methyl group of orally administered AAF. The somewhat larger amounts of radioactivity accounted for in the body of the rat outside the gastrointestinal tract after oral administration of 9-C^{14}-labeled AAF, compared with the recoveries of the diazotizable material, may possibly be due to the greater sensitivity of their test. But it seems to be more reasonable to conclude that the nitrogen from the administered AAF is present in the tissues in a nondiazotizable form and/or in extremely water-soluble material. Since a different distribution in the body of the 9-labeled carbon and the nitrogen of the molecule is possible, the results of studies now in progress with AAF containing N^{15} will be a most important means for following the metabolism of this carcinogen.

SUMMARY

The diazotization method of Westfall, with slight modifications, was used to determine the presence of 2-acetylaminofluorene (AAF), 2-aminofluorene (AF), or of closely related derivatives in acetone extracts of the tissues and in ether extracts of hydrolysates of protein and lipid fractions of the tissues of rats, following single doses of AAF by stomach tube or after repeated feeding of the carcinogen.

The method of analysis yields reliable recoveries of AAF from acetone extracts of tissues to which AAF is added before homogenization, but not more than 5 per cent of that which disappeared from the gastrointestinal tract during 5 to 23 hours of fasting could be recovered from the acetone extracts of the tissues and excreta of treated rats.

The recovery of 300 μg. of AAF added before the saponification of fat was excellent, but saponification of extracts of lipid material from rats' tissues did not increase the amount recovered over that from unsaponified lipid extracts, except where large amounts of fat interfered with the glacial acetic acid solution of the carcinogen.

Moderate to large losses of AAF were encountered in control recovery experiments with the procedures used in the acid or alkaline hydrolyses of protein material. With 300 μg. of AAF added to the control tissues the poorest recovery, however, and that with only two fractions of tissues, was 75 μg. (25 per cent), whereas the hydrolysates of protein material from the experimental rats yielded completely negative results. Tryptic digests of protein material from the tissues of treated rats also failed to give any color in the test, although pilot control recovery tests demonstrated almost quantitative recovery.

It is concluded that orally administered AAF does not combine as such or as AF with the proteins or saponifiable lipids of the tissues, at least, in combinations that are reversible upon hydrolysis, or in amounts that represent significant fractions of that part of the administered AAF which cannot be recovered from acetone extracts of the tissues and excreta of the rats.

The experimental results indicate that much of the administered AAF is changed by the tissues of the rat into a nondiazotizable form.

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The Distribution of S^{35} from Labeled DL-Methionine in Mice Bearing Carcinoma of the Breast, Neoplasms of the Hematopoietic System, or Liver Abscesses*

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The experiments on the distribution of radiosulfur reported here were intended to give empirical information on the relative uptake of the isotope by neoplastic tissue, as compared to normal tissue, in order to evaluate chances for a localized radiation therapy of tumors with labeled methionine. The possibility of an accumulation of radioactive sulfur in neoplasms, after administration of the isotope in an amino acid, was suggested by reports that neoplasms take up amino acids more rapidly than do normal adult tissues (24) and by the evidence for an increased metabolic turnover rate in tumors (4, 17). It was realized, however, that other factors, such as increased permeability or increased retention of metabolites, might account for any concentration of radiosulfur in the tissues (11). Some preliminary data concerning the distribution of S^{35} from labeled methionine in mice bearing mammary carcinomas or transplanted ependymoblastomas have been published from this laboratory (9). We have since found that varying amounts of S^{35} are lost by the method of tissue preparation which was used in this preliminary work. Some of our earlier experiments have been therefore repeated with more dependable technical procedures. In addition, we have extended our studies to neoplasms of the hematopoietic organs and liver abscesses. The results reported now are similar to those obtained in the preliminary investigations: in both studies the concentration of radiosulfur, at the times chosen for analysis, was higher in tumor tissue than in many normal tissues, but lower than in liver, kidney, spleen, or the intestines.

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METHODS

DL-Methionine-S^{35} was prepared from thiourea-S^{35} (2). The purity and identity of the compound were checked by paper chromatography with *n*-butanol and phenol. The amino acid was located on the paperogram by the ninhydrin reaction, and the isotope by scanning with a monitor and by radioautography on x-ray film. Spraying ninhydrin on the papers produced but one spot, whose R_f value agreed with that reported in the literature for DL-methionine (16) and with that of a concurrently run authentic sample of the compound. All the radioactivity was found to be localized within the ninhydrin spot.

All radioactivity measurements were carried out on barium sulfate. The organic materials were digested by the Carius method (7, 19, 23), and enough sodium sulfate-S^{32} was added to the digest to yield a total precipitate of 170–180 mg. barium sulfate. The barium sulfate was filtered onto stainless steel planchets having a surface area of 1.77 $cm.^2$ and clad with Whatman No. 50 filter paper (1). Care was taken to obtain a smooth mat of barium sulfate with even thickness. The precipitate was washed with water and ethanol, air-dried, and counted with a thin window bell-type Geiger tube. The counting rates were corrected for background, coincidence, and decay in the customary manner (15). For urinary sulfate-S^{35} determinations, digestion by the Carius procedure was omitted.

Radioactivity assays on individual tissues and tissue fractions were run in duplicate or triplicate wherever sufficient material was available. The values for the S^{35} concentrations were reproducible to within ± 5 per cent in 46 tissues, to within ± 10 per cent in 16 tissues, to within ± 20 per cent in 9 tissues, and to within ± 33 per cent in 5 tissues. The reproducibility was generally better for

tissues with high isotope concentrations, such as liver, than for tissues with low isotope concentrations, such as muscle. Some of the discrepancies may have been due to inhomogeneity of the tissues, so that what was thought to be a duplicate sample from a tissue actually had a different radiosulfur concentration.

Proteins were obtained by dispersing the tissues in a glass homogenizer with 10 per cent aqueous trichloroacetic acid; the homogenate was centrifuged, and the residue washed 4 times with 10 per cent trichloroacetic acid, 3 times with ethanol, and then thrice with ethyl ether. Finally, the residue was dried *in vacuo* over phosphorus pentoxide at room temperature; it was considered the "protein" fraction for our purposes (3). The wet tissue minus this "protein" fraction was designated as the "nonprotein" fraction; the latter comprised water and the solutes which were removed from the wet tissues by treatment with aqueous trichloroacetic acid, ethanol, and ethyl ether.

The distribution of S^{35} was studied in the tissues of eleven mice.[1] Two animals served as normal controls, two had mammary carcinomas, two carried transplanted lymphosarcomas, two had liver abscesses, two had lymphatic leukemia, and one had myelogenous leukemia. Further details concerning these animals are given in Table 1.

The animals received intraperitoneal injections of an aqueous solution of labeled DL-methionine containing 20–40 μc. of S^{35}, and were decapitated 24 hours later. It was assumed that administration of the labeled compound in a single dose, rather than continuous dosage, would favor selective uptake. We studied the distribution of the isotope at 24 hours, rather than at some other interval, because our preliminary experiments led us to expect a maximum concentration of the isotope in the tumors at this time. Also, the data of Maas (10), Friedberg (3), and Tarver (21) seemed to suggest that the distribution of radiosulfur 24 hours after its administration would approximate the relative distribution of the isotope retained in the tissues over most of its biological life. Up to the time of the experiment, the mice were on an *ad libitum* fox chow diet, but they were put on a fast after the administration of the amino acid. No untoward effects were observed from the injection.

TABLE 1

MICE USED IN THE STUDY OF S^{35} DISTRIBUTION

Exp. no.	Pathology	Wt. of mouse (gm.)	Age (mo.)	Sex	Methionine* dose	Weight of organs (in per cent of body weight) Liver	Kidney	Spleen	Lung	Heart	Brain	Tumor	Remarks†
1	Normal control	26.5	8	F	0.6	4.2	1.3	0.26	0.47	0.40	0.96		A.K.; strain Strong A
2	Normal control	34.0	12	M	1.6	3.7	1.1	0.28	0.78	0.34	0.93		A.K.; strain Strong A
3	Mammary carcinoma	40.0	8	F	1.3	5.1	1.4	0.21	0.51	0.35	0.93	8.2	J.J.B.; strain Z Ce F_1; lactating
4	Mammary carcinoma	33.0	11	F	1.6	4.0	1.4	0.45	0.51	0.31	0.84	2.0	J.J.B.; strain Strong A
5	Transplanted lymphosarcoma	20.5	2	M	1.3	3.9	1.0	0.43	0.55	0.36	1.6	5.8	A.K.; line 1016; 1st transfer of spontaneous lymphosarcoma which had originated in this line
6	Transplanted lymphosarcoma	19.5	2	M	2.0	2.7	0.96	0.25	0.39	0.29	1.6	8.4	A.K.; line 1016; 5th transfer of spontaneous lymphosarcoma which had originated in this line
7	Liver abscess	26.0	?	F	0.6	9.1	1.4	1.1	0.88	0.36	1.4		G.M.; strain Strong A; fed N-acetylaminofluorene
8	Liver abscess	26.0	?	M	0.6	6.9	1.3	1.8	1.2	0.38	1.1	10.0	G.M.; strain Strong A; fed N-acetylaminofluorene
9	Myelogenous leukemia	26.0	?	M	0.6	6.8	1.7	4.3	1.3	0.99	1.2		A.K.; line 15; 49th transfer
10	Lymphatic leukemia	17.5	2	M	1.5	5.1	1.1	2.8	1.3	0.43	1.6	1.4	A.K.; line 676; 30th transfer
11	Lymphatic leukemia	27.0	23	M	0.2	5.9		0.87	0.55	0.35		6.0	A.K.; strain CBA; had received 200 r x-ray at age 1 mo.

* Mg. DL-methionine injected per gram body weight.

† The notations A.K., J.J.B., and G.M. indicate mice from the colonies of Drs. A. Kirschbaum, J. J. Bittner, and George Moore, respectively.

[1] The mice were obtained from the colonies of Drs. A. Kirschbaum, J. J. Bittner, and George Moore, to whom we wish to express our appreciation for their co-operation in this study.

RESULTS AND DISCUSSION

The concentration of S^{35} in the wet tissues (Table 2, roman type) and protein and nonprotein fractions (Table 3) is expressed in terms of fraction of total counts per minute administered per mg. sample × weight of mouse in milligrams. This expression normalizes values obtained in animals of various weights and injected with various amounts of the isotope to animals of unit size and injected with unit doses of labeled methionine. The percentage of the total dose in the various whole tissues is also given (Table 2, figures in italics).

TABLE 2

CONCENTRATION AND DISTRIBUTION OF S^{35} IN TISSUES 24 HOURS AFTER INTRAPERITONEAL INJECTION OF DL-METHIONINE-S^{35}

Exp. no.	Pathology	Liver	Both kidneys	Spleen	Stomach*	Small intestine†	Large intestine†	Lung	Whole blood	Heart	Brain	Muscle	Tumor
1	Normal control	0.67	0.50	0.44	0.43			0.30	0.29	0.27	0.11	0.07	
		2.8	*0.67*	*0.11*				*0.14*		*0.11*	*0.10*		
2	Normal control	1.1	0.84	0.62	0.97	1.4	0.79	0.34	0.29	0.33	0.16	0.11	
		4.2	*0.94*	*0.17*				*0.27*		*0.11*	*0.15*		
3	Mammary carcinoma	0.74	0.58	0.55	0.55	0.82	0.51	0.39	0.16	0.25	0.14	0.07	0.33
		3.8	*0.82*	*0.11*				*0.20*		*0.07*	*0.13*		*2.7*
4	Mammary carcinoma	1.2	0.82	0.87	0.96	1.3	0.92	0.55	0.43	0.65	0.18	0.10	0.69
		4.7	*1.1*	*0.39*				*0.28*		*0.20*	*0.15*		*1.4*
5	Transplanted lymphosarcoma	1.2	0.79	0.66	0.69	0.93	0.72	0.47	0.22	0.30	0.16	0.17	0.65
		4.7	*0.81*	*0.28*				*0.25*		*0.11*	*0.26*		*3.8*
6	Transplanted lymphosarcoma	0.88	0.52	0.47		0.89	0.66	0.36	0.16	0.13	0.10	0.04	0.51
		2.3	*0.50*	*0.12*				*0.14*		*0.04*	*0.16*		*4.2*
7	Liver abscess	1.2	0.79	0.90	0.75			0.51	0.30	0.45	0.19	0.14	0.13‡
		11.0	*1.1*	*0.94*				*0.44*		*0.16*	*0.27*		
8	Liver abscess	1.1	0.80	1.1	0.73	1.2		0.55	0.36	0.43	0.19	0.23	0.16‡
		7.6	*1.0*	*2.0*				*0.65*		*0.16*	*0.21*		*1.6*
9	Myelogenous leukemia	0.80	0.57	1.0	0.71			0.55		0.34	0.16	0.12	
		5.5	*0.98*	*4.4*				*0.71*		*0.34*	*0.19*		
10	Lymphatic leukemia	0.84	0.61	0.85	0.63	0.83	0.67	0.34	0.20	0.24	0.14	0.16	0.72
		4.3	*0.70*	*2.4*				*0.46*		*0.10*	*0.22*		*0.98*
11	Lymphatic leukemia	1.4		1.1				0.72	0.38	0.49			1.0
		8.2		*0.95*				*0.39*		*0.17*			*6.1*

* Glandular portion only.

† Wall only.

‡ Abscess, purulent material.

All figures except those in italics represent the concentration in $\frac{\text{fraction of total counts per minute administered}}{\text{mg. tissue}} \times$ weight of mouse in mg.

Figures in italics represent distribution in per cent dose in organ.

TABLE 3

DISTRIBUTION OF S^{35} BETWEEN PROTEIN AND NONPROTEIN FRACTIONS OF LIVERS, MUSCLES, AND TUMORS

	Experiment no.								
	2	3	4	5	6	7	8	10	11
Liver:									
Protein content, per cent	24	21	21	21	23		15		19
Concentration of S^{35} in protein fraction	3.3	2.3	3.3	3.1	2.5		3.3		4.7
Concentration of S^{35} in nonprotein fraction*	0.46	0.31	0.58	0.71	0.40		0.72		0.62
S^{35} in protein fraction, per cent of total S^{35} in liver*	69	66	61	53	64		45		64
Muscle:									
Protein content, per cent	20	24	22	20		13	18	21	
Concentration of S^{35} in protein fraction	0.25	0.18	0.26	0.40		0.36	0.38	0.44	
Concentration of S^{35} in nonprotein fraction*	0.075	0.032	0.049	0.11		0.11	0.20	0.090	
S^{35} in protein fraction, per cent of total S^{35} in muscle*	44	64	60	47		32	29	56	
Tumor:									
Protein content, per cent		17		9.5	13				12
Concentration of S^{35} in protein fraction		0.81		3.5	2.5				4.8
Concentration of S^{35} in nonprotein fraction*		0.23		0.35	0.20				0.53
S^{35} in protein fraction, per cent of total S^{35} in tumor*		42		51	65				54

* Calculated from protein content and concentration of S^{35} in wet tissue and protein.

Concentrations in $\frac{\text{fraction of total counts per minute administered}}{\text{mg. sample}} \times$ weight of mouse in mg.

The concentration of S^{35} was highest in the liver and intestines; on the average, decreasing concentrations were found in the kidneys, spleen, intestinal wall, stomach, lung, whole blood, heart, brain, and muscle. The concentration of radiosulfur in the tumors was higher than its concentration in most normal tissues, but lower than that in the intestines, livers, kidneys, or spleens. In contrast to its concentration in the tumors, the concentration of the isotope in both abscesses was lower than in any of the tissues in the same animal. Grossly normal lactating mammary gland tissue showed a lower concentration (0.18) than homologous tumor tissue (0.33) in the same animal (Experiment No. 3). The spread of the values for the concentration of the isotope in a given organ among various animals was of the order of $\pm\frac{1}{2}$ of the average value. While this may appear to be a large variability, it is no greater than that of the ratio of organ to body weight.

A comparatively large fraction of the dose was present in the liver, as one would expect from the high concentration in this organ and from its size. The percentage of the dose found in the spleen varied by a factor of 40 from animals with normal-sized spleens to animals with enlarged spleens. However, it appeared that the large amounts of S^{35} found in the spleens in Exp. Nos. 8, 9, and 10 were not due to the mass of the organs alone, since the concentration of the isotope in the spleens tended to increase with their relative size. A slightly higher percentage of the dose was found in the lungs of mice with leukemia or liver abscesses than in the lungs of the other animals. The percentage of the dose in the tumors and the tumor weights given in Table 1 represent minimum values only, since it is likely that small metastatic and infiltrating lesions were missed at autopsy.

The protein fractions of the livers, tumors, and muscles, although they comprised only one-tenth to one-fifth of the mass of the tissues, contained one- to two-thirds of the total amount of radiosulfur in the tissues, and the concentration of the isotope in the protein fractions was 2–12 times greater than in the nonprotein fractions. The ratio of the total amount of radiosulfur in the protein fraction to the total amount of radiosulfur in the nonprotein fraction was relatively constant, despite large differences in the concentration of the isotope between various tissues. The concentration of radiosulfur in the protein fraction from lymphatic tumors was equal to or greater than its concentration in the protein fraction from the livers of the same animals (Exp. Nos. 5, 6, and 11). The fact that the concentration of the isotope in the wet tumor tissue of these animals was lower than in the wet liver tissue may be due to the low protein content of the tumors. Our experiments do not permit one to draw conclusions regarding the chemical form in which the radiosulfur was present in the tissues and tissue fractions; however, one may surmise from what is known about sulfur metabolism (5) that appreciable amounts of radiosulfur retained in the animals were no longer incorporated in methionine.

Within the 24-hour period of our experiments the animals excreted up to 10 per cent of the injected methionine sulfur in the stools. An average of 47 per cent appeared in the urine, the value in individual animals ranging from 15 to 90. Inorganic sulfate accounted for 62–99 per cent of the isotope in the urine.

Our values for the percentage of methionine sulfur excreted in the urine are comparable to corresponding values in the literature (12, 13, 20, 22), obtained from balance experiments with methionine doses of 0.1–1 mg/gm body weight. If one assumes the normal methionine level in serum (about 10^{-2} mg/ml) (6, 18) to be representative of the normal methionine level in the body water, and on the premise that the body water accounts for about 60 per cent of the body weight, it seems likely that the amount of methionine which we injected into our animals may have raised the methionine concentration in their body fluids by a factor of 100. Following the administration of much smaller amounts of labeled methionine (10^{-2}–10^{-3} mg/gm body weight), Kinsell (8), Reed (14), and Tarver (21) found about 10 per cent of the radiosulfur in the urine within the first 24 hours. Comparison of our data with those of Kinsell (8), Tarver (21), and Friedberg (3) suggests, as far as comparison of the data is possible, that even a hundredfold change in methionine dosage has only a relatively slight effect on the percentage of methionine sulfur excreted, or on the distribution of the isotope in the tissues.

SUMMARY

The distribution of S^{35} in mice bearing mammary carcinomas, lymphosarcomas, leukemias, or liver abscesses was studied 24 hours after an intraperitoneal injection of DL-methionine-S^{35}. The concentration of the isotope was highest in the intestinal wall and liver, lowest in muscle; it was considerably higher in protein fractions than in nonprotein fractions of the tissues. The concentration of S^{35} in the wet tumor tissue was lower than that in the wet liver tissue of the same animals; but the concentration of the isotope in the proteins of lymphosarcomas was equal to or greater than

that in the liver proteins from the same animal. The fact that the S^{35} in wet lymphosarcoma tissue is lower than in wet liver tissue is probably due to the low protein content of lymphosarcomas. In contrast to that in tumors, the concentration of S^{35} in liver abscesses was very low. On the premise that the concentration of a radioactive isotope in tumor tissue must be much higher than in other tissues of the body if the isotope is to be useful in the treatment of neoplastic diseases, the data reported here do not favor methionine-S^{35} as a possible therapeutic agent for mammary carcinomas, lymphosarcomas, or leukemias.

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Carcinogenic Hydrocarbons and Related Compounds in Processed Rubber*

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During the construction of a chemical apparatus in which ground glass connections could not be used because of the large size of the flasks, it was found that benzene extracts of rubber stoppers previously washed with ether and methanol still contained blue-green fluorescing substances. These extracts had the ultraviolet absorption spectra of carcinogenic hydrocarbons and other compounds of interest. This discovery seemed of such great possible importance to investigators in cancer research, to the rubber industry, and to public health that further experiments were made. It was found that extracts of rubber stoppers were carcinogenic for mice. In similar extracts, the presence of pyrene, 3,4-benzpyrene, chrysene, and other aromatic hydrocarbons was detected. The same compounds were found in benzene extracts of an automobile tire. Further observations, together with comments on the possible origins and importance of these compounds, are here presented.

BIOLOGICAL TESTS FOR CARCINOGENICITY

Methods.—Rubber stoppers of different color and consistency were washed with cold ether and extracted in a Soxhlet apparatus with hot, redistilled benzene for 1 hour. The resulting reddish-brown extract showed a strong blue-green fluorescence. A second extraction gave a colorless but fluorescent solution. The two extracts were combined, and the solvent was distilled off on a steam bath. The reddish-brown, partially crystalline residue was weighed and used for biological testing in mice.

This rubber extract was tested for carcinogenicity by skin painting and by subcutaneous injection. The tests were all made in young mice of C57 black strain and of both sexes. All lesions and many relatively normal-appearing painted skins were studied on histological section.

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Results of skin painting experiments.—The rubber extracts were dissolved in benzene and applied by dropper to the skin of the interscapular region 3 times each week. A total of 199 paintings was made in 544 days. One hundred mice were exposed, half of them to 15 per cent and the other half to 2 per cent solutions. The experiments were terminated at the end of 20 months by examination at autopsy of all survivors.

With the higher concentration, the mice surviving 12 months received approximately 500 mg. of extract; those surviving 18 months were treated with about 1,000 mg. The mice painted with the dilute solution were exposed to 80 mg. of extract in 12 months and 160 mg. in 18 months.

Epilation occurred within 2 weeks. In the mice dying early in the experiment because of infection and other intercurrent causes, a definite sequence of changes was seen in microscopic sections. These were, first, atrophy of the epidermis and of the epithelial appendages of skin, infiltration of mast cells into the dermis, and hyperkeratosis, followed by hypertrophy of the epidermis with parakeratosis. Finally, tumors, both papillomas and carcinomas, developed.

The first tumor was seen on the 252d day, and others followed soon after (Table 1). Many mice had multiple (up to six) tumors, some of which were transient and others permanent and progressive. Carcinomas were first recognized in the eleventh month by increased firmness, infiltration at the base, and ulceration (Figs. 1, 2), and they were later verified on microscopic section (Figs. 3–6). At 12 months, when 23 mice were alive in the experiment with the 15 per cent rubber extract solution, 10 had tumors. The total per cent tumor yield was 45.7 with the 15 per cent, and 6.7 with the 2 per cent extracts. The per cent yield was calculated as the number of mice dying with tumor among those alive in the experiment when the first tumor was seen.

Benzene controls were treated in the same way, except that the number of paintings had to be re-

duced to two per week midway in the experiment because of its high toxicity. No tumors were seen in these mice.

Results of injection experiments.—One hundred and fifty mice received subcutaneous injections of rubber extracts in tricaprylin as solvent. Different dose levels were used in three experiments of 50 mice each, in attempts to circumvent loss of the extract by extrusion due to its caustic action. No successful test for carcinogenicity of the material was ever possible. In the first experiment the dose per mouse was 200 mg. of rubber extract made up to 0.2 ml. in tricaprylin; in the second experiment the amount injected was 50 mg. in 0.1 ml. tricaprylin; in the third it was 10 mg. in 0.05 ml. of the solvent. Almost every mouse developed a slough at the injection site between the first and third weeks. Subsequent regular examinations failed to disclose injection site masses, and at necropsy little or no injected chemical was seen.

TABLE 1

CARCINOGENIC EFFECTS OF RUBBER EXTRACTS

PAINTING MATERIAL	NO. MICE	INDUCTION TIME FIRST TUMOR (DAYS)	SURVIVAL AND RESULTS (MONTHS) 0	9	12	15	18	20	NO. TUMOR MICE Papillomas	Carcinomas	Total	PER CENT YIELD
Rubber extract, 15 per cent	Survivors	252	50	35	23	14	7	3				
	Dead with tumor					3	6	7	4	12	16	45.7
Rubber extract, 2 per cent	Survivors	376	50	41	30	26	10	8				
	Dead with tumor							2	2	0	2	6.7
Benzene controls	Survivors		72	46	35	18	17	12				
	Dead with tumor								0	0	0	0.0

Mouse survival was as follows: 68 were alive at 12 months, 45 at 15 months, 33 at 18 months, and 25 at the end of 20 months when the experiments were terminated. No tumors were found at the sites of injection, but this result is not considered significant. It is concluded that no adequate test for carcinogenic activity of the rubber extract was made by the injection method.

CHEMICAL STUDIES

Rubber stoppers of six types.—Rubber stoppers, sizes 3–5, were washed with ether, separated into six groups on the basis of their color and density (black, white, brown, red, gray, light-brown), and weighed. Approximately 100 gm. of rubber stopper of each color was extracted for 20 minutes as described above. The extracts were evaporated. They yielded approximately 0.5 per cent of a mixture of oils and crystals. From each extract, 22 fractions were obtained on chromatography through columns of activated aluminum oxide with increasing proportions of ether in petroleum ether as eluant. All fractions were evaporated on a hot water bath, transferred to 50-ml. Erlenmeyer flasks with ether, and the solvent was evaporated under a stream of nitrogen. Each fraction with ether as a solvent was analyzed for its absorption spectrum in a Beckman ultraviolet spectrophotometer between the wave lengths of 220 and 440 mμ.

A qualitative comparison of the constituents of these extracts revealed that hydrocarbons with ultraviolet absorption spectra of interest in this study were present in all six extracts. Characteristic absorption spectra indicated the presence of cyclopentenophenanthrene, pyrene, and a closely related compound (Charts 1, 4, and 7). The spectra of other fractions devoid of the characteristics of aromatic hydrocarbons were not further investigated.

Black rubber stoppers.—In another extraction of rubber stoppers on a large scale, 82 gm. of residue was obtained from 4 kg. of black rubber stoppers. This extract was divided into 27 fractions by chromatography. Further purification of significant fractions was attempted by the following methods: isolation of the insoluble picrates following Jones's procedure (11), purification of the fractions by cold concentrated sulfuric acid extraction (3), sublimation under high vacuum, and repeated chromatography and crystallization.

The same three spectra previously mentioned were detected in this extract. In addition, five other characteristic absorption spectra were found. The most important compound detected was 3,4-benzpyrene (Chart 3), a strong carcinogen. In addition, crystals of chrysene were isolated and identified (Chart 2). A number of other characteristic absorption spectra were observed, whose identity has not yet been completely determined. They are tentatively described as alkyl pyrene (Chart 7), 1,12-benzperylene (Chart 5), 1,2-benzpyrene (Chart 6), and possibly a 1,2-benzanthracene derivative (Chart 8). Thus, in this extract the absorption spectra of eight aromatic hydrocarbons were detected (Chart 9).

Automobile tire.—To extend this work to other rubber goods, an automobile tire was cut into

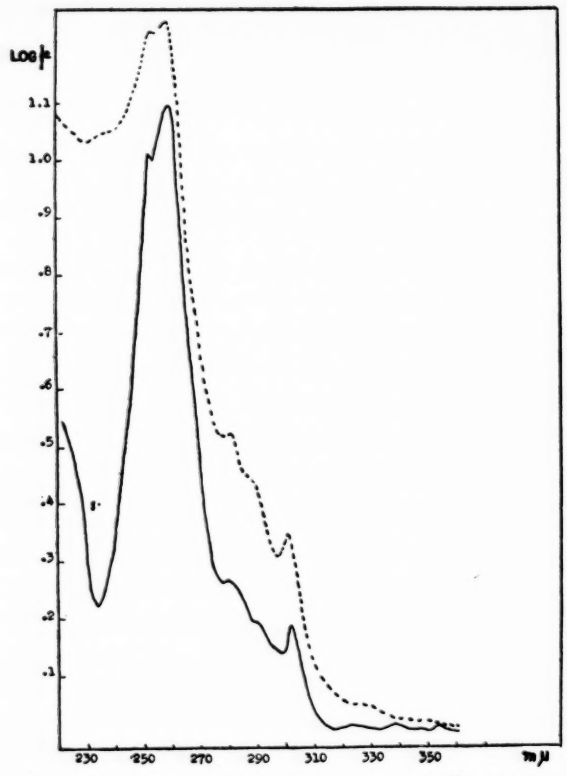


CHART 1. ——— = Absorption spectrum of methyl cyclopentenophenanthrene which is obtained by selenium dehydrogenation of cholesterol.

----- = Absorption spectrum of alkyl cyclopentenophenanthrene isolated from processed rubber.

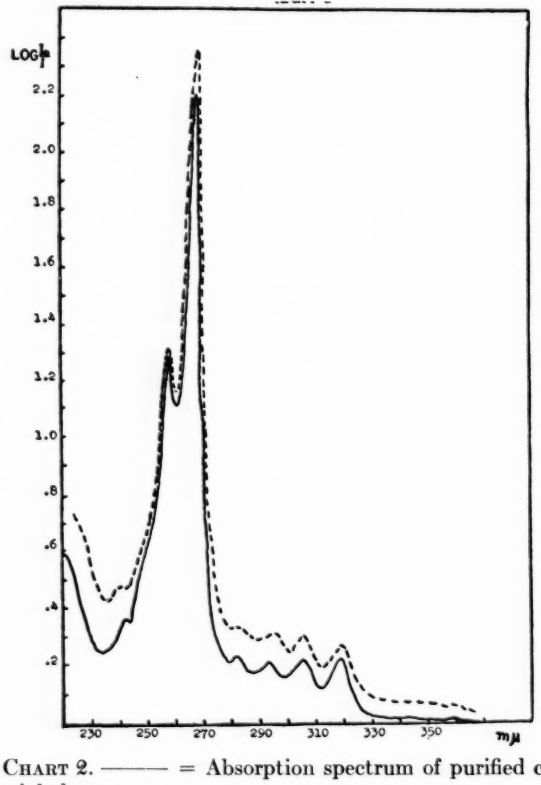


CHART 2. ——— = Absorption spectrum of purified commercial chrysene.

----- = Absorption spectrum of chrysene isolated from processed rubber.

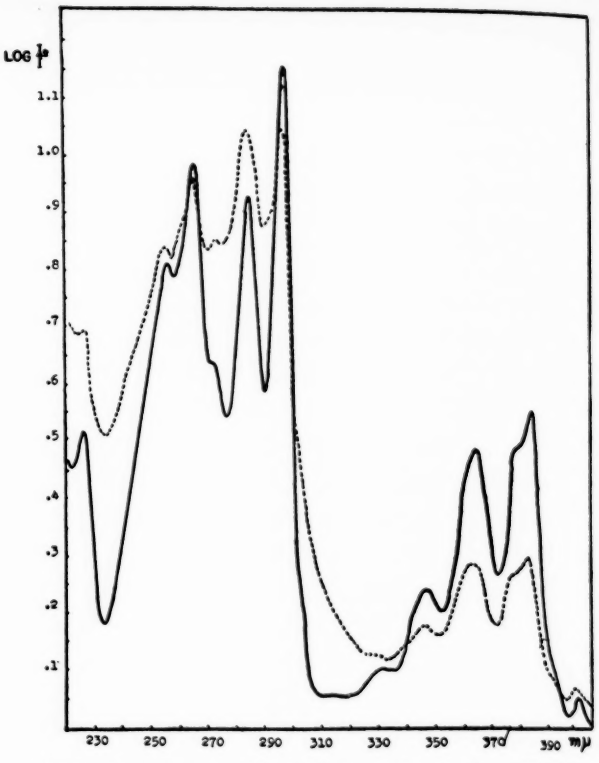


CHART 3. ——— = Absorption spectrum of purified commercial 3,4-benzpyrene.

----- = Absorption spectrum of 3,4-benzpyrene isolated from processed rubber.

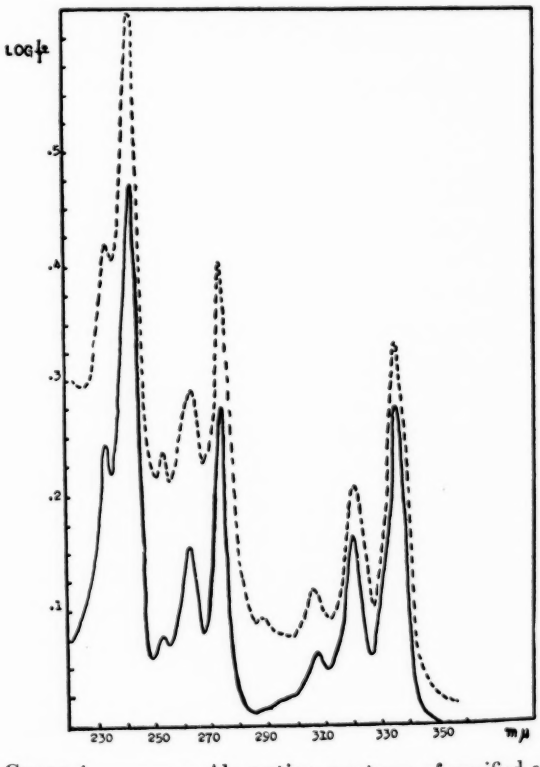


CHART 4. ——— = Absorption spectrum of purified commercial pyrene.

----- = Absorption spectrum of pyrene isolated from processed rubber.

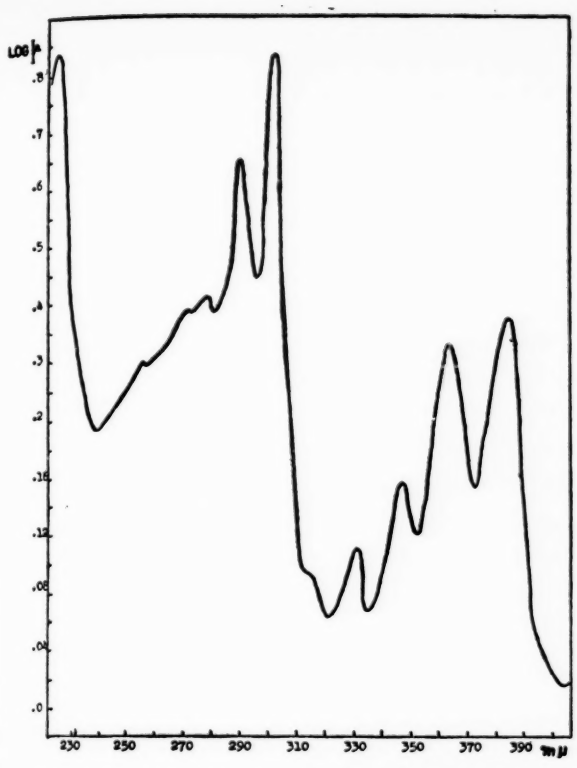


CHART 5.—Absorption spectrum of crystals isolated from processed rubber, and tentatively identified as 1,12-benzperylene.

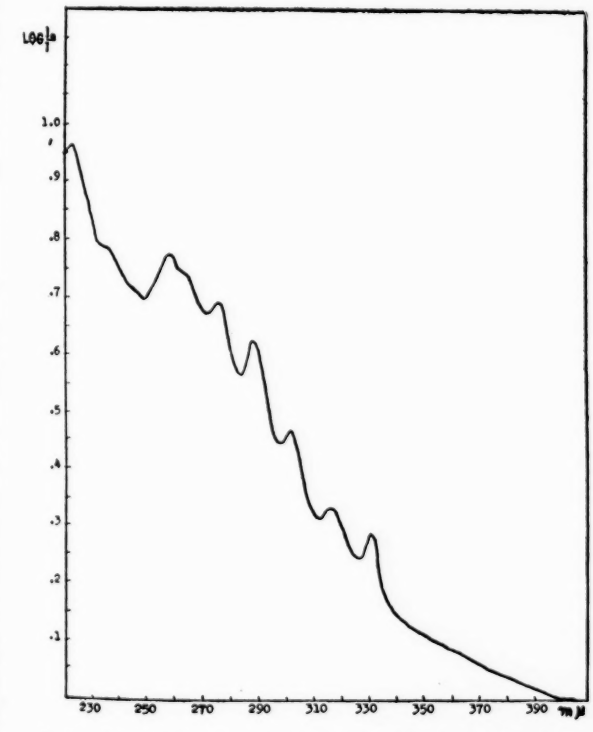


CHART 6.—Absorption spectrum of a fraction isolated from processed rubber and tentatively identified as 1,2-benzpyrene.

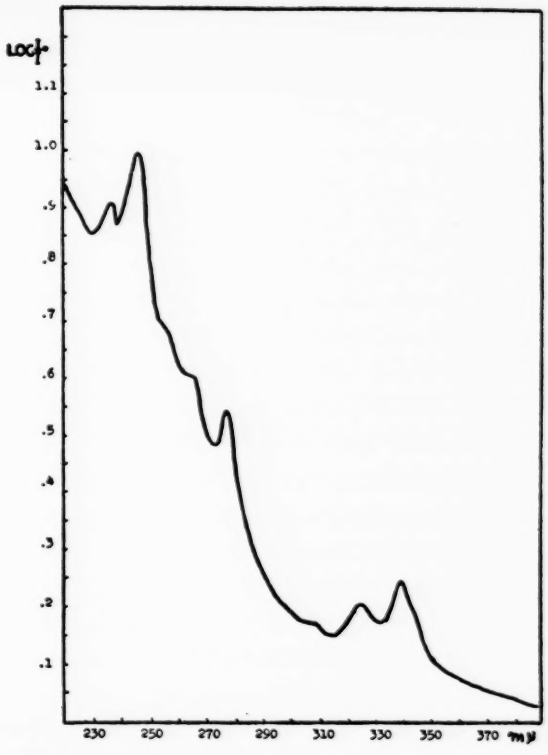


CHART 7.—Absorption spectrum of a compound isolated from processed rubber and tentatively identified as mono- or dialkyl pyrene.

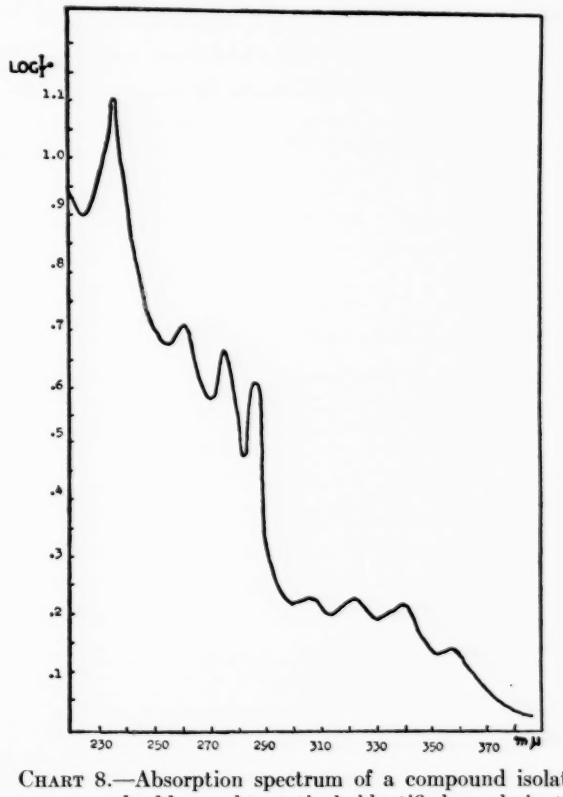
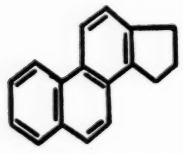
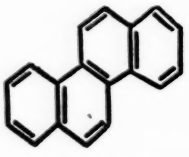


CHART 8.—Absorption spectrum of a compound isolated from processed rubber and tentatively identified as a derivative of 1,2-benzanthracene.

small squares and treated like the rubber stoppers described previously. Two kg. of the rubber tire (in which the nonrubber fabric was included) were extracted. Forty fractions were obtained by chromatography. Exactly the same compounds were eluted from the absorption column and in the same sequence as in the black rubber stopper experiment. These data are shown in Table 2.

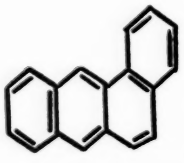


Cyclopentenophenanthrene

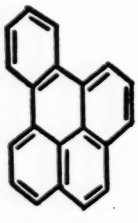


Chrysene

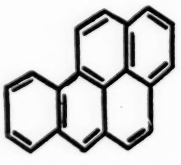
Pyrene



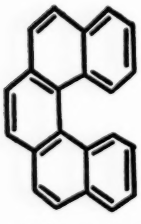
1,2-benzanthracene



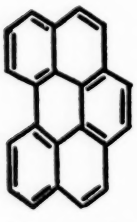
1,2-benzpyrene



3,4-benzpyrene



3,4,5,6-dibenzphenanthrene



1,12-benzperylene

CHART 9.—Structural formulae of compounds detected in extracts of rubber stoppers and an automobile tire by their absorption spectra.

Identification of compounds.—Detection of aromatic hydrocarbons becomes easier and identification more accurate as the number of condensed aromatic rings in the molecule increases, because the absorption maxima shift to longer wave lengths, where few of the accompanying impurities absorb energy. Standard identification may be difficult, because only minute quantities of the hydrocarbon may be present in quantities of material which are suitable for ultraviolet absorption spectroscopy. Considerable reliance has been therefore placed on the latter procedure.

The compounds reported in this paper were identified by their ultraviolet absorption spectra which, in every case possible, were compared with spectra of commercially available samples (Charts 1–4) and with published reports of others (5, 6, 10, 15). Where possible, melting points and mixed melting points of the compound or of its derivatives were made. By these methods the following compounds were identified: 3,4-benzpyrene, pyrene, and chrysene were identified beyond reasonable doubt; cyclopentenophenanthrene and 1,12-benzperylene were identified, although no information is available on substituent groups; 1,2-benzpyrene, alkyl pyrene, and the 1,2-benzanthracene derivative were least pure, and their identification as such is not certain.

3,4-Benzpyrene was detected by its absorption maxima between 340 and 410 mμ. It was purified by extraction with cold concentrated sulfuric acid followed by chromatography. Only minute quantities were obtained. The absorption spectrum was identical with that of commercially available purified 3,4-benzpyrene (Chart 3).

From the same fraction that contained the 3,4-benzpyrene, a sulfuric acid-insoluble compound was obtained which was tentatively identified as *1,2-benzpyrene* by its absorption spectrum. As pure 1,2-benzpyrene was not available for comparison, this oil could not be further identified.

Pyrene was identified by its characteristic absorption spectrum, which resembled that of commercially available pyrene (Chart 4). Crystalline pyrene picrate was formed from this material as well as from commercial pyrene. Both gave sharp melting points at 222°–223° C.; the melting point was not depressed by mixing the compounds.

Chrysene was isolated from impurities by taking advantage of its insolubility in cold solvents. It was identified by its characteristic absorption spectrum which was like that of a commercial product (Chart 2). It was purified by recrystallization from hot benzene. Its melting point was 248°–249° C. No depression of melting point occurred after it was mixed with commercial chrysene.

Cyclopentenophenanthrene was detected by its absorption spectrum, which was identical with that of methyl cyclopentenophenanthrene (Chart 1). It was a colorless, blue-fluorescing oil which could not be further purified by crystallization, sublimation, picrate formation, or repeated chromatography. It was probably a homologue or a mixture of homologues of methyl cyclopentenophenanthrene.

1,12-Benzperylene is the tentative identification of square plate-like crystals whose absorption spectrum is shown in Chart 5. Their melting point

was 249°–250° C. Their spectrum is identical with that given by Clar for 1,12-benzperylene (5), except that all maxima are shifted by 2 mμ to the ultraviolet. It is possible that the absorption spectrum illustrated by us is that of 3,4,5,6-dibenzphenanthrene, which is not available for comparison.

The compound designated as a *pyrene derivative* (Chart 7) possessed the spectrum of pyrene with a bathochromic shift of 5 mμ, indicating the presence of a substituent group on the resonating aromatic system. It cannot, however, be identified with 3- or 4-methyl pyrene, because its maxima are shifted midway between the absorption peaks of these two compounds.

Both products used in these experiments (rubber stoppers and automobile tire) contained processed rubber. Unprocessed rubber, natural or synthetic, was not studied by us, although others have done so by spectrophotometry. The absorption spectra which they reported have not been those of any known carcinogens (2, 9, 12). Carcinogens are, therefore, most probably introduced during processing of rubber. A number of theoretical possibilities exist by which this might be done. They might be introduced with the catalysts, the pigments, the anti-oxidants, or the fillers (1, 16).

Mercaptobenzothiazole and tetramethylthiuram disulfide are typical catalysts. They are sulfur and nitrogen-containing compounds which have not been tested for carcinogenicity. They are, however, not structurally related to any known carcinogen. The pigments are both organic and inorganic; some of the former fall into the category of the azo dyes. The dyes used are not identical with the known carcinogenic azo dyes, but, on the other hand, they have not been tested. Some of the anti-oxidants are related to β-naphthylamine, a known carcinogen. They have been inactive on tests for carcinogenicity (4, 17).

Among the fillers, the most widely used material is carbon black, which is similar to soot, a substance recognized as carcinogenic since 1775 (Percivall Pott). However, a recent survey of a large carbon black industry by Ingalls (8) revealed no unusually great incidence of cancer among the workers in that industry. Hueper (7) described the potential dangers to workers in the carbon black and rubber industries without, however, reporting cases of cancer. A real laboratory test, either chem-

TABLE 2

COMPARISON OF THE CHROMATOGRAMS OF EXTRACTS OBTAINED FROM RUBBER STOPPERS AND AN AUTOMOBILE TIRE

Fractions			
Rubber stoppers	Rubber tire	Absorption maxima	Chemical nature
1, 2	1–5	230, 275, 325	naphthalene derivative
3	6		mixture
4, 5	7–9	252, 258, 278, 288, 300	phenanthrene derivative
6	10		mixture
7	11–12	232, 240, 252, 262, 272, 286, 305, 319, 334	pyrene
8a	13a	236, 262, 276, 286, 308, 324, 342, 358	1,2-benzanthracene derivative(?)
8b	13b	236, 246, 258, 266, 278, 325, 340	pyrene derivative
9–11	14–16		mixture
12, 13	17–19	265	?
14–16	20–22	242, 258, 268, 296, 306, 320, 340, 344, 360	chrysene
17	23–25		mixture
18a–20a	26a–29a	226, 256, 266, 274, 284, 296, 330, 346, 364, 384, 404	3,4-benzpyrene
18b–20b	26b–29b	224, 236, 262, 278, 288, 302, 316, 332	1,2-benzpyrene(?)
21	30–32		mixture
22	33–35	222, 254, 270, 276, 288, 300, 314, 330, 346, 362, 384, 406	1,12-benzperylene(?)
23	37, 38	245, 272, 302, 310	?
24–27	36, 39, 40	285	?

The spectrum shown in Chart 8 has not been identified. It may be that of an impure derivative of 1,2-benzanthracene.

COMMENTS

Benzene extracts of rubber stoppers induced papillomas and carcinomas when painted on the skin of mice. Similar extracts made from rubber stoppers and an automobile tire possessed the absorption spectra of a number of aromatic hydrocarbons. One of them is known as a strong carcinogen (3,4-benzpyrene) and three others as weak carcinogens (chrysene, pyrene, and 1,2-benzpyrene). The other compounds have not yet been tested for carcinogenic potency, but from their chemical configuration they may be expected to be carcinogenic. With the presence of four known carcinogens and other possible carcinogens the demonstrated activity of the rubber extracts is not surprising.

ical or biological, of the possible carcinogenic hazards in carbon black has not come to our attention.

A great variety of carbon blacks exist, differing in their method of preparation and resulting particle size (1). Gas black is made by the incomplete combustion of natural gas. Furnace black is also produced from natural gas by a regulated access of air. These conditions resemble those used by Kennaway in the production of carcinogens from acetylene (13, 14). Lamp black is obtained by the incomplete combustion of oils and tars. It might contain carcinogens formed during manufacture, as well as those previously present in the oils and tars.

We can only speculate on the significance of our findings. The carcinogens are present in small amounts, they are relatively insoluble, and they are, in part, relatively inaccessible to contact exposure, being imbedded in rubber. They may be harmless under ordinary conditions of exposure. However, when some organic solvents are used, as under laboratory conditions, extraction is easy, and the danger of accidental, unrecognized contamination during experiments in cancer research is great.

The consumer public has contact with many products containing carbon black. In addition to those already mentioned may be listed inner tubes, conveyor belts, shoe soles and heels, rubber hose, flooring, and molded goods in the rubber industry. The newspaper and paint industries are other important users of carbon black. We know of no evidence that exposure to these products constitutes a practical hazard.

The greatest hazard would appear to be to workers in the rubber and carbon black industries. However, perusal of the literature on cancers in these industries appears to disclose no undue incidence. It is possible that the dangers are minimized by the use of modern protective measures. It is also possible that there is no practical danger because of the inaccessibility of these compounds to contact exposure.

SUMMARY

1. A benzene extract of rubber stoppers was carcinogenic by skin painting in mice.

2. From such extracts of rubber stoppers and of an automobile tire, eight aromatic hydrocarbons were isolated. 3,4-Benzpyrene, pyrene, chrysene, and alkyl cyclopentenophenanthrene were identified beyond reasonable doubt by their ultraviolet absorption spectra, and, additionally, in the case of pyrene and chrysene by melting point determinations. The identity of another crystalline product, 1,12-benzperylene, seems fairly certain. The oily nature of three products, 1,2-benzpyrene, alkyl pyrene, and alkyl 1,2-benzanthracene made definite identification as yet impossible.

3,4-Benzpyrene, 1,2-benzpyrene, pyrene, and chrysene have been described as carcinogenic in the literature. The other four compounds are structurally related to carcinogenic compounds.

3. The possible importance of these observations to workers in the industries concerned, to the general public, and to cancer research has been discussed.

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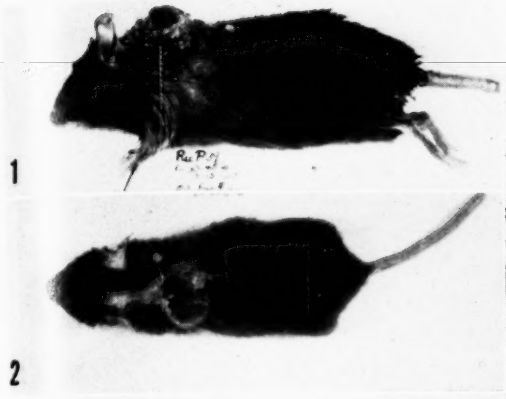

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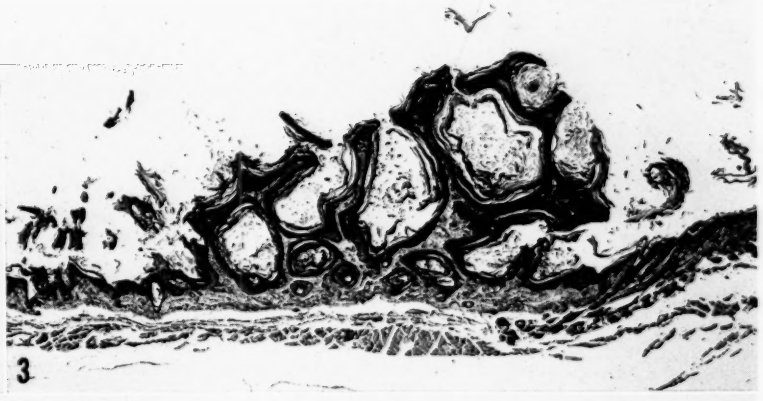

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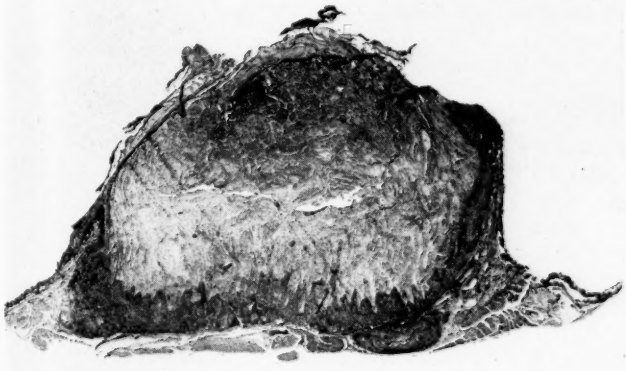

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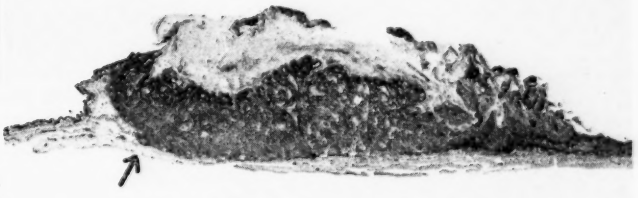

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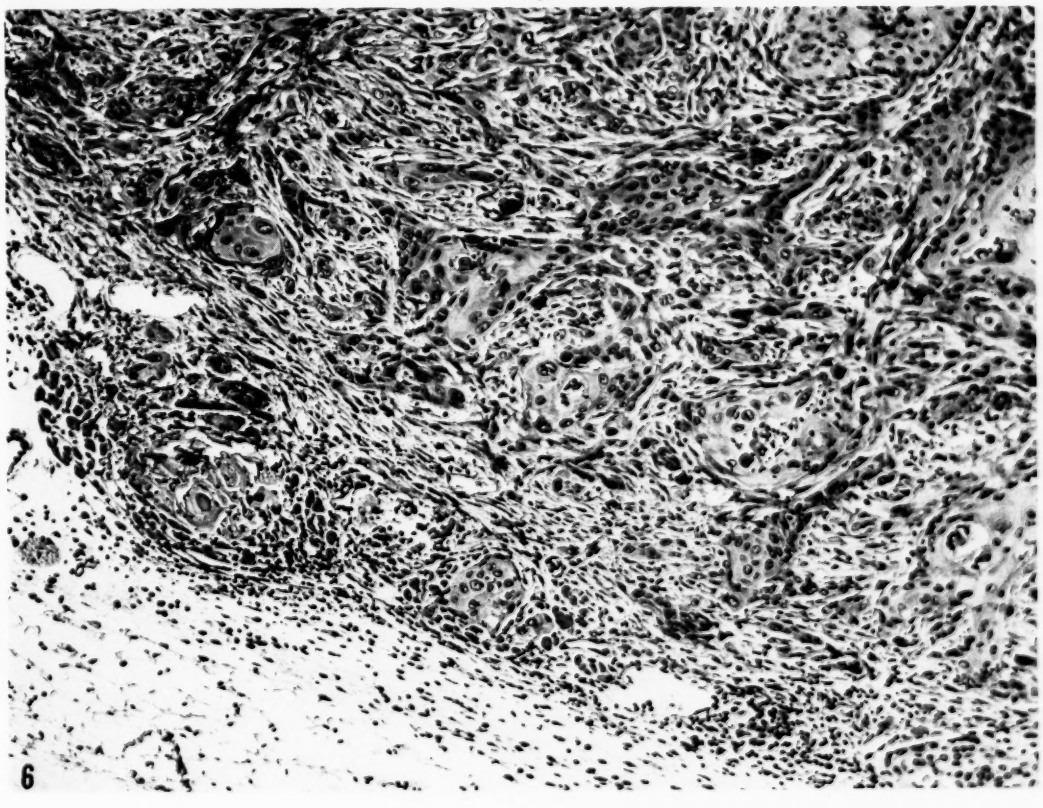

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FIG. 1.—Squamous-cell carcinoma weighing 1.5 gm. in a mouse dying 561 days after the beginning of the experiment. The top is horny; the adjacent skin is elevated by lateral subcutaneous extensions of the tumor. Painted with the 15 per cent extract of rubber stoppers.

FIG. 2.—Ulcerated, flat squamous-cell carcinoma near a small papilloma in a mouse painted with 15 per cent rubber stopper extract. Death at 20 months. Photomicrograph of this tumor shown in Figures 5 and 6.

FIG. 3.—Skin papilloma (×20) in a mouse painted with 15 per cent extract of rubber stoppers. Mouse died of pneumonia 384 days after the first painting, having also another skin papilloma.

FIG. 4.—Squamous-cell carcinoma (×4) in a mouse painted with 15 per cent extract of rubber stoppers. The tumor consists of a mass of kerato-hyaline with anaplastic cells at the margins infiltrating through the superficial muscle.

FIG. 5.—Photomicrograph (×5) of the squamous-cell carcinoma shown in Figure 2. Arrow points to area enlarged in Figure 6.

FIG. 6.—Photomicrograph (×120) showing a squamous-cell carcinoma invading superficial muscle (center left) and subcutaneous tissues. This is a higher power of the area indicated by the arrow in Figure 5, and photographed in Figure 2.

Roentgen Irradiation of Desoxyribonucleic Acid

III. The Relation of Dose to Degree of Depolymerization *in Vivo* and the Influence of Oxygen Tension

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The mechanism of the action of roentgen irradiation in cell destruction was undertaken with the thought that if the mechanism were known, it might be modified so that malignant tissue would be more readily destroyed than the surrounding normal tissue. Cell destruction may partly be due to the depolymerization of desoxyribonucleic acid (DNA), as was suggested earlier (9). DNA is believed to be depolymerized by a chemical indirect effect—that is, by free radicals generated from the cellular water surrounding the DNA. Support for this theory was found in the inhibition by thiourea of depolymerization induced by a dose of 1,000 roentgens. That depolymerization occurred 24 hours after irradiation, but not immediately, offered additional support.

Doses smaller than the 1,000 r reported earlier (9) have been investigated and have been found to depolymerize DNA *in vivo*. The degree of depolymerization, as measured by the streaming birefringence and the structural viscosity of the DNA solutions, has been found to be proportional to the dosage.

The oxygen concentration of the atmosphere during irradiation was shown to influence the susceptibility of mice (7) and rats (2) to irradiation. An atmosphere of low oxygen concentration was found by Baker and Sgourakis (1) to reduce the number of x-ray-induced mutations in Drosophila. Similarly, when the atmosphere of air was replaced with nitrogen, the frequency of aberrations in Tradescantia was found by Giles and Riley (3) to be reduced, whereas the frequency was increased when the air was replaced with oxygen. When air was replaced with nitrogen, the resistance of bacteria to irradiation was increased, according to Hollaender *et al.* (5).

The effects of an atmosphere of 10 per cent oxygen and of 100 per cent oxygen during the irradiation of rats have been investigated. It has been found that the depolymerization of DNA was inhibited by an atmosphere of 10 per cent oxygen and that the depolymerization of DNA was not increased by an atmosphere of 100 per cent oxygen, except in a few cases. The involution of rat thymus tissue induced by x-radiation has been investigated, and it has been found that it was inhibited more effectively by thiourea than by a reduced oxygen atmosphere.

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MATERIALS AND METHODS

The apparatus and methods used to determine the structural viscosity and streaming birefringence of the DNA solutions were described earlier (9), but the birefringence apparatus was modified slightly to make it more sensitive.

Male albino rats, weighing 125–180 gm., from the Albino Farms, Red Bank, N.J., which had been fed a diet of Fox Food Blox (Allied Mills), were irradiated in groups of three or four, with the same cage arrangement described earlier (7) for the irradiation of mice. A wire screen divided the top half of the cage from the bottom half to prevent overlying of the animals and also to provide an even distribution of the gas mixture above them. The gas (a mixture of 10 per cent O_2 and 90 per cent N_2 or 100 per cent O_2) was administered for 7 minutes before irradiation and during the entire period of irradiation. The constants of the x-ray machine were 250 kv. and 15 ma. A 0.5-mm. copper filter was used in addition to the inherent filtration of 3 mm. of aluminum. The irradiation rates for 600 r, 350 r, and 250 r were 77 r/min, 74 r/min, and 78 r/min, respectively.

The DNA solutions were prepared from the pooled thymus tissue of three or more rats according to a modified Mirsky-Pollister procedure (9). The pH of all solutions used in the preparation of DNA was buffered at 10.75, with the exception of the 0.14 M NaCl solution (pH 6.85) used to remove the ribonucleic acid. The concentrations of

the DNA solutions were based on the concentration of phosphorus.

To determine the effect of thiourea on the x-ray-induced involution of rat thymus, aqueous thiourea solution (9 gm/100 ml) was injected intraperitoneally ½ hour before irradiation (2,000 mg/kg). The thymus tissues were removed and weighed 24 hours after irradiation.

lar to that reported by Greenstein and Jenrette (4). Depolymerization of the DNA was observed in all ten solutions prepared from rats irradiated with 600 r; the mean decrease in the ν_∞ was 32.2 per cent and in the streaming birefringence, 60.7 per cent. Depolymerization was obtained with 350 r in all but the first group (Table 2). Depolymerization in all groups was obtained when

TABLE 1

STRUCTURAL VISCOSITY, ν_∞, AND STREAMING BIREFRIGENCE OF DNA SOLUTIONS* FROM RAT THYMUS 24 HOURS AFTER X-RADIATION *in Vivo* WITH 600 r

	Controls			Irradiated			Decrease	
Conc. (mg P/ml)	N/P	ν_∞	Str. brfrg. (mm.)	N/P	ν_∞	Str. brfrg. (mm.)	ν_∞ (per cent)	Str. brfrg. (per cent)
0.0726	2.01	2,381	210	2.12	1,470	150	38.3	28.5
0.0596	2.29	3,430	390	2.27	1,730	180	49.5	53.8
0.0396	1.92	3,100	180	1.97	2,210	60	28.7	66.75
0.0394	2.21	2,620	180	2.23	1,510	60	42.4	66.75
0.0380	1.57	3,600	180	2.18	2,300	70	36.0	61.0
0.0300	1.71	3,419	120	1.77	2,063	45	40.0	62.0
0.0270	1.93	3,350	90	2.67	2,480	60	26.0	33.33
0.0165	2.38	4,560	90	2.36	3,080	25	32.5	72.30
0.0150	1.57	3,500	45	2.60	3,270	15	6.6	62.66
0.0142	1.88	3,940	35	2.25	3,080	0	22.0	100.00
						Mean	32.2	60.7

* Five rat thymus tissues per solution.

TABLE 2

STRUCTURAL VISCOSITY, ν_∞, AND STREAMING BIREFRINGENCE OF DNA SOLUTIONS* FROM RAT THYMUS 24 HOURS AFTER X-RADIATION *in Vivo* WITH 350 r

	Controls			Irradiated			Decrease	
Conc. (mg P/ml)	N/P	ν_∞	Str. brfrg. (mm.)	N/P	ν_∞	Str. brfrg. (mm.)	ν_∞ (per cent)	Str. brfrg. (per cent)
0.030	1.70	2,300	150	1.73	2,300	150	0.0	0.0
0.029	2.14	2,900	210	2.07	1,565	90	46.0	57.0
0.030	2.19	2,950	210	1.89	2,250	120	24.0	43.0
0.030	2.07	3,070	240	1.86	2,250	90	27.0	62.0
0.025†	1.87	3,280	210	1.72	1,745	60	47.0	72.0
0.030	1.91	2,360	180	1.63	1,650	60	30.0	67.0
0.030	2.15	2,360	150	1.77	1,830	90	22.5	40.0
0.030	1.93	2,050	120	1.64	1,710	90	16.6	25.0
0.030‡	1.82	2,900	270	1.93	1,950	120	32.8	55.5
0.030	2.00	2,950	210	2.07	2,200	120	25.4	42.8
0.030	1.97	2,300	150	1.71	1,650	90	28.3	40.0
0.030	1.93	2,710	180	2.02	2,010	120	25.8	33.3
	Mean	2,623	188.1		1,942	103.6	25.2	42.3

* Three rat thymus tissues per solution.
† Not included in means.
‡ Six rat thymus tissues per solution.

RESULTS

Depolymerization of DNA in vivo *by 600 r, 350 r, and 250 r.*—DNA was found to be depolymerized 24 hours after irradiation with either 600 r, 350 r, or 250 r. It may be seen from Table 1 that when the concentration of the DNA solutions decreased, the degree of streaming birefringence decreased, while the degree of structural viscosity, ν_∞, generally increased. This inverse relationship is similar to the linear relationship obtained by Fricke and Morse

250 r was used (Table 3). The mean decrease in the ν_∞ and the streaming birefringence for the four doses (1,000 r [9], 600 r, 350 r, 250 r) was converted to the ν_∞ and streaming birefringence remaining and plotted against the roentgen doses (Chart 1). The ν_∞ and streaming birefringence remaining were found to be proportional to the roentgen dose. These curves are comparable to the

as quoted by Lea (6) in the oxidation of ferrous sulfate by roentgen irradiation.

Effect of 10 per cent oxygen and of 100 per cent oxygen on the roentgen-ray depolymerization of DNA.—From the results shown in Table 4 it may be seen that 10 per cent oxygen–90 per cent nitrogen administered before and during irradiation inhibited the depolymerization of DNA in all but two groups. The mean increases due to 10 per cent oxygen were 13.69 per cent and 37.5 per cent for ν_∞ and streaming birefringence, respectively. Depolymerization was increased in only five groups out of eleven when 100 per cent oxygen was used (Table 5). However, the differences between the means of the control and the 100 per cent oxygen groups for ν_∞ and streaming birefringence were very small, so that it can be said that 100 per cent oxygen was ineffective, as compared to 10 per cent oxygen, in changing the degree of depolymerization of DNA.

Effect of 10 per cent oxygen and of thiourea on the x-ray-induced involution of rat thymus tissue.—The mean weight of fresh thymus (mg/100 gm body weight) was found to be 202 for the control rats, 111 for the rats irradiated under normal oxygen tension (20 per cent oxygen), 126 for the rats irradiated under reduced oxygen tension (10 per cent oxygen), and 164 for the rats injected with thiourea before irradiation (Table 6). The difference between the mean of the group irradiated under normal oxygen tension and the mean of the group irradiated under a reduced oxygen tension was not statistically significant, whereas the difference between the mean of the irradiated group and the mean of the irradiated thiourea-treated group was significant.

DISCUSSION

Depolymerization of DNA *in vivo* has been observed when a dose as small as 250 r was used. It is possible that even smaller doses would depolymerize DNA *in vivo*. According to Sparrow, chromosome aberrations and mitotic inhibition by ionizing irradiation may both be due to the action of the irradiation on the nucleoprotein complex of the chromosomes or genes (10). The mechanism of this action also may be indirect, mediated through free radicals generated from the surrounding water.

An examination of the data revealed that the degree of depolymerization of DNA *in vivo* was

TABLE 3

STRUCTURAL VISCOSITY, ν_∞, AND STREAMING BIREFRINGENCE OF DNA SOLUTIONS* FROM RAT THYMUS 24 HOURS AFTER X-RADIATION *in Vivo* WITH 250 r

	Controls			Irradiated			Decrease	
Conc. (mg P/ml)	N/P	ν_∞	Str. brfrg. (mm.)	N/P	ν_∞	Str. brfrg. (mm.)	ν_∞ (per cent)	Str. brfrg. (per cent)
0.025	1.72	2,480	150	1.70	1,770	90	28.6	40.0
0.025	1.92	2,940	150	1.86	2,170	90	26.2	40.0
0.025	1.76	2,580	150	1.77	2,310	120	10.5	20.0
0.025	1.59	1,960	90	1.67	1,745	60	11.0	33.3
0.025	2.28	2,240	120	2.09	1,885	60	15.9	50.0
0.025	1.83	2,590	240	1.91	2,165	150	16.4	37.5
0.025	1.62	2,380	150	1.96	2,090	90	12.2	40.0
0.025	1.82	2,870	210	1.76	2,090	120	27.2	42.8
0.025	2.01	2,590	210	1.70	2,380	150	8.1	28.6
0.030†	2.12	2,720	240	1.99	1,825	120	33.2	50.0
0.030†	2.01	2,540	240	1.89	2,120	180	16.5	25.0
	Mean	2,514	163.3		2,067	103.3	17.3	36.9

* Three rat thymus tissues per solution.
† Not included in means (four rat thymus tissues per solution).

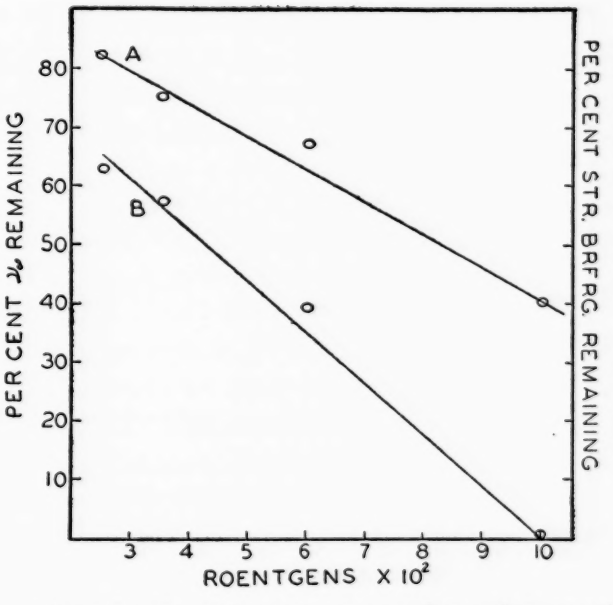

CHART 1.—*A:* relation between x-ray dose and structural viscosity, ν_∞, remaining. *B:* relation between x-ray dose and streaming birefringence (Str. Brfrg.) remaining.

TABLE 4

STRUCTURAL VISCOSITY, ν_∞, AND STREAMING BIREFRINGENCE OF DNA SOLUTIONS* FROM RAT THYMUS 24 HOURS AFTER X-RADIATION *in Vivo* UNDER 10 PER CENT O_2

		20 PER CENT O_2 (NORMAL) IRRADIATED			10 PER CENT O_2 IRRADIATED			INCREASE	
Conc. (mg P/ml)	X-ray dose (r)	N/P	ν_∞	Str. brfrg. (mm.)	N/P	ν_∞	Str. brfrg. (mm.)	ν_∞ (per cent)	Str. brfrg. (per cent)
0.025	350	1.72	1,745	60	1.68	2,030	90	16.3	50.0
0.030†	350	1.63	1,650	60	1.58	1,880	60	13.95	0.0
0.030†	250	1.70	1,770	90	2.40	2,480	150	40.2	66.7
0.025	250	1.96	2,170	90	2.07	2,660	120	22.6	33.3
0.025	250	1.77	2,310	120	1.80	2,580	150	11.75	25.0
0.025	250	1.67	1,745	60	1.75	1,960	90	12.3	50.0
0.025	250	2.09	1,885	60	1.90	1,680	60	0.0	0.0
0.025	250	1.91	2,165	90	1.76	1,815	90	0.0	0.0
0.025	250	1.96	2,090	90	1.71	2,425	150	16.0	66.7
0.025	250	1.76	2,090	120	1.83	2,730	210	30.6	75.0
		Mean	2,025	86.2		2,235	120	13.69	37.5

* Three rat thymus tissues per solution.
† Not included in means.

TABLE 5

STRUCTURAL VISCOSITY, ν_∞, AND STREAMING BIREFRINGENCE OF DNA SOLUTIONS* FROM RAT THYMUS 24 HOURS AFTER X-RADIATION *in Vivo* UNDER 100 PER CENT O_2

		20 PER CENT O_2 (NORMAL) IRRADIATED			100 PER CENT O_2 IRRADIATED			DECREASE	
Conc. (mg P/ml)	X-ray dose (r)	N/P	ν_∞	Str. brfrg. (mm.)	N/P	ν_∞	Str. brfrg. (mm.)	ν_∞ (per cent)	Str. brfrg. (per cent)
0.030	350	1.71	1,650	90	1.71	1,770	90	0.0	0.0
0.030	350	1.70	2,300	150	1.70	1,535	90	33.3	40.0
0.030	350	2.07	1,565	90	1.83	1,800	90	0.0	0.0
0.030	350	2.02	2,010	120	1.83	1,595	90	20.7	25.0
0.030	350	1.86	2,250	90	1.59	1,890	60	16.0	33.3
0.030	350	1.72	1,745	60	1.68	1,815	90	0.0	0.0
0.030	350	1.63	1,650	60	1.70	2,000	60	0.0	0.0
0.030†	350	1.93	1,950	120	1.63	1,830	90	6.15	25.0
0.029‡	350	2.10	2,650	240	1.93	2,170	180	18.10	25.0
0.030§	250	1.99	1,825	120	1.93	2,170	180	0.0	0.0
0.030§	250	1.87	2,120	180	1.97	2,120	210	0.0	0.0
		Mean	1,907	108		1,848	105	7.62	12.33

* Three rat thymus tissues per solution.
† Six rat thymus tissues per solution.
‡ Not included in means.
§ Four rat thymus tissues per solution.

TABLE 6

EFFECT OF 10 PER CENT O_2 AND OF THIOUREA ON INVOLUTION OF RAT THYMUS 24 HOURS AFTER X-RADIATION

No. of rats	Treatment	Range of rat weights (gm.)	Av. rat weight (gm.)	Mg thymus/100 gm body wt	
				Mean ($\bar{x}$)	Standard deviation*
25	none	125–212	158	202	34.33
31	250 r	120–200	150	111	35.26
31	10 per cent O_2–250 r	120–184	145	126	45.41
15	thiourea-250 r	116–176	148	164	49.86

* $S = \sqrt{\frac{\Sigma (x - \bar{x})^2}{N - 1}}$ where N = number of rats .

directly proportional to the roentgen-ray dose. It may also be seen that the dose that depolymerized DNA *in vivo* by 50 per cent (average of curves *A* and *B*, Chart 1) was about 650 r. Since the LD_{50} for rats irradiated with 250 kv. is about 650 r, this relation suggests that the degree of depolymerization of DNA is an index of the mortality of rats. Furthermore, it was pointed out by Sparrow (11) that the "amount of chromosome fragmentation is a fair index of cell lethality." Depolymerization of DNA should therefore be considered an important factor in cell destruction by ionizing irradiation.

The inhibition of the x-ray-induced depolymerization of DNA *in vivo* by decreasing the oxygen tension during irradiation is further evidence that this effect of irradiation is due to the interaction of DNA and highly reactive free radicals such as OH, H and O_2H, as well as hydrogen peroxide generated by the irradiation of water surrounding the DNA. A low oxygen tension would tend to decrease the formation of the free radicals and hydrogen peroxide (7). It is difficult, however, to visualize the depolymerization of DNA by free radicals in the presence of other cellular constituents that could protect DNA by competing for the free radicals unless DNA were more sensitive to free radicals. It appears that DNA is much more sensitive *in vivo* than *in vitro*, since a dose of only 250 r is sufficient to depolymerize DNA in the former case, while a much larger dose is necessary in the latter case (8, 12). Furthermore, the possibility should not be overlooked that DNA does not actually exist in a highly polymerized form *in vivo*, but is simply polymerized by the procedures employed for its extraction. The action of roentgen irradiation mediated through free radicals can then be considered to interfere with the mechanism by which DNA is polymerized.

SUMMARY

The degree of depolymerization of DNA extracted from rat thymus 24 hours after roentgen irradiation *in vivo*, as measured by the structural viscosity and streaming birefringence of the solutions, was found to be directly proportional to doses of 250 r to 1,000 r.

A reduced oxygen tension (10 per cent) was found to inhibit the depolymerization of DNA, whereas an increased oxygen tension (100 per cent) was found to be relatively ineffective in increasing the depolymerization.

A 10 per cent oxygen tension was found to be less effective than thiourea in preventing the involution of rat thymus tissue induced by roentgen irradiation.

These results are regarded as further evidence for the indirect mechanism of the action of roentgen irradiation *in vivo*.

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The Carbohydrate Metabolism of the Brown-Pearce Carcinoma of the Rabbit in Normal and Hypoglycemic Serum*

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INTRODUCTION

The observation of Warburg, Wind, and Negelein (15) that the tumor tissue metabolism of tumor-bearing animals kept in insulin convulsions for several hours was not affected stimulated many other investigators to inquire into the relationship of substrate concentration to oxygen consumption of tumors (1, 4–7, 13). All these investigators were in agreement in showing a higher consumption of oxygen in the absence of glucose than in its presence. The largest difference (50 per cent) was reported by Burk *et al.* (4) for chicken tumors.

The present study was concerned with two principal aspects of the metabolism of carbohydrate in the Brown-Pearce epithelioma of the rabbit; namely, the relationship of serum sugar level and the ratio of lactic acid production to oxidation in tumor slices.

Only primary growths were used. They were removed from the testis at 14–28 days after inoculation. Fifteen to 30 mg. of the slices, prepared with a straight razor and kept in Ringer phosphate solution, buffered to pH 7.35 until ready for study, were suspended in 2 ml. of rabbit's serum during a determination.

Sera were prepared from rabbits which had been on a fast for 14–24 hours. Fifteen to 30 ml. of blood, an amount which depended on the number of flasks employed, were taken from the starved animal for the preparation of normal serum. The rabbit then received subcutaneous injections of 12 units regular insulin, and, when the pre-convulsive sugar level was reached, as manifested by a characteristic behavior, or when frank convulsions occurred, the animal was bled for preparation of the hypoglycemic serum.

Barcroft-Brody manometers with flasks designed by Marsh (9) were used for the determination of respiration. Lactic acid values were determined colorimetrically with the Klett-Summerson photoelectric colorimeter, according to the procedure of Barker and Summerson (2). Glucose determinations were made on zinc filtrates of the sera by the Somogyi (11) method.

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RESULTS

Experiments performed after the manner of Warburg, Wind, and Negelein (15), with Ringer-phosphate medium free of CO_2 and glucose, showed that hypoglycemia in the tumor-bearing animal did not affect the rate of oxidation. In a comparison of sera from normal animals with sera from the same animals in insulin shock (of a degree which would certainly produce a difference in glucose level), an average increase of 17.2 per cent (eleven animals) was found in the Q_{O_2} values from the tumor slices in the latter media over those in the former.

In six experiments on fortifying shock sera with 66–138 mg. per cent of glucose, the stimulating effect of shock sera on oxygen consumption was completely nullified. In six other experiments the addition of 50 and 100 mg. per cent of glucose to normal sera had no effect on Q_{O_2} values.

The trend of the data assembled from the experiments just described is summarized with approximate accuracy in Chart 1.

First, it was necessary to prove that the tumors metabolizing at a higher rate were affected in the same proportion by a change in the serum glucose as those metabolizing at a low rate. From the group of eleven experiments mentioned above, the average ratio of the higher range Q_{O_2} values in hypoglycemic sera to those in normal sera was 1.16. A similar average ratio of lower range Q_{O_2} values to those in normal sera was 1.19. The difference is within the limits of experimental error.

Since it was shown that the metabolic rate of a tumor is not a factor in determining its response to glucose for two widely differing serum glucose levels, it was assumed that the same condition holds for intermediate serum glucose levels. That a given

tumor had a high or a low energy turnover was in every instance determined by measuring its Q_{O_2} in normal serum as a base line; as shown above, its Q_{O_2} would be high or low in the same proportion at any other glucose level within the range of the graph. The formula used in adjusting Q_{O_2} values is shown below.

$$Bcx = Bx\left[\frac{(1/N)\Sigma(A_1 + A_2 + \ldots)}{Ax}\right].$$

Ax = Q_{O_2} of any tumor in normal serum.
N = number of determinations.
Bx = Q_{O_2} found for same tumor in serum altered, up or down, with respect to glucose.
Bcx = corrected Q_{O_2} in altered serum.
$\frac{1}{N}\Sigma(A_1 + A_2 + \ldots) = 5.01$ (mean of 11 exps.).

Chart 1 shows a graph of corrected Q_{O_2} values plotted against the glucose level of the serum in which the respiration was studied. Sugar levels indicated by the squares were determined directly. Triangles indicate the glucose levels were estimated, based on average values for normal and for shock sera of 140 mg. per cent and 36 mg. per cent, respectively.

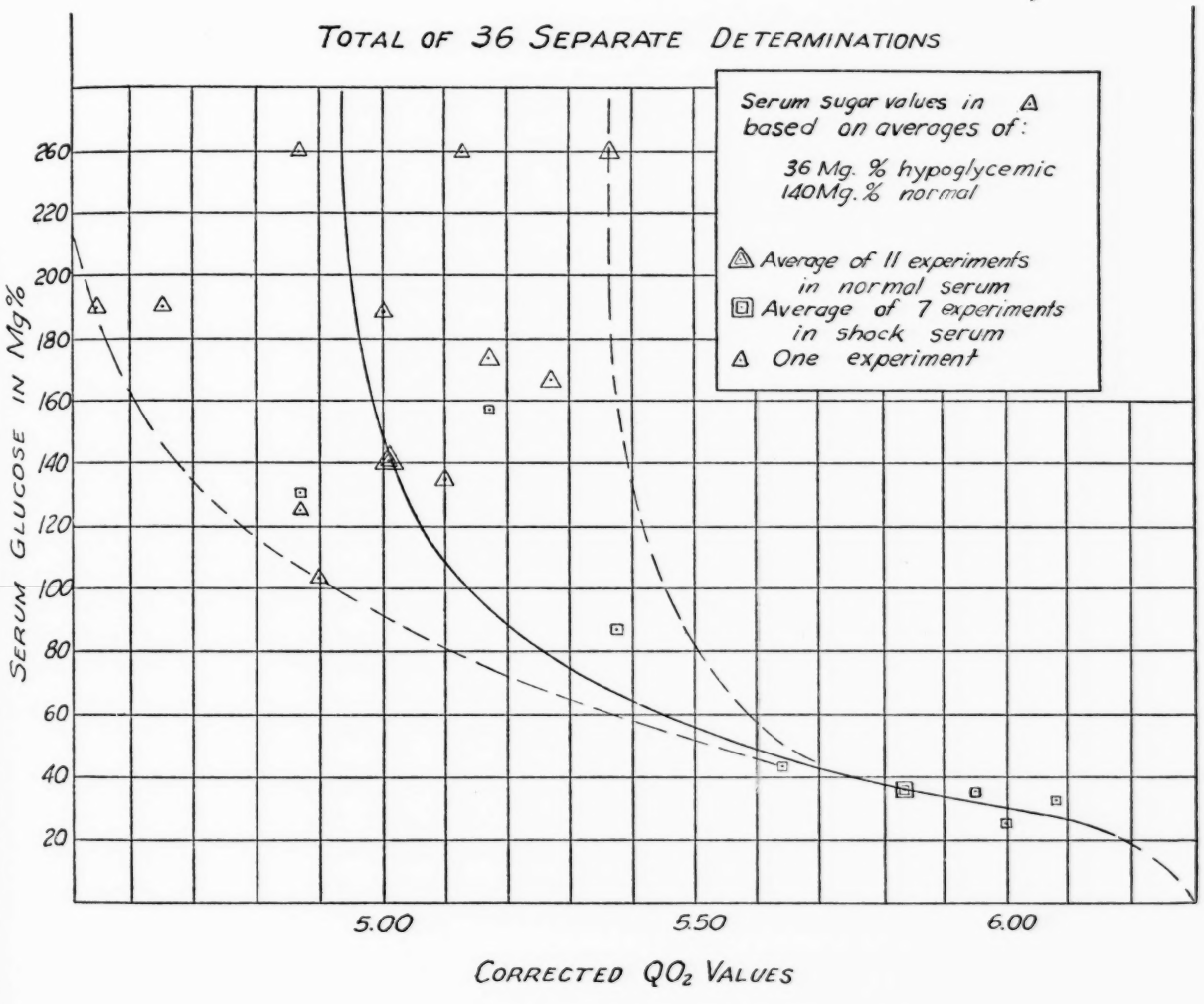

CHART 1

Although the wide spread of points at the higher glucose levels prevents accurate localization of the curve in this region, that it should follow a course nearly paralleling the glucose axis is indicated by the results which showed that glucose added to normal serum did not affect Q_{O_2} values.

Aerobic glycolysis.—Warburg, Wind, and Negelein (15) perfused tumors with arterial blood and observed that the tumor cells abstracted 57 per cent of the sugar from it, whereas in normal tissues the sugar lost from the blood ranged between 2 and 18 per cent. As long as adequate oxygen was supplied to normal tissues, none of the sugar was utilized for production of lactic acid, but tumors produced from 46 to 49 mg. of this acid for each

100 ml. of blood. However, when the sugar concentration is reduced by insulin shock, the formation of lactic acid is also reduced, as may be seen in Table 1.

It is evident that, whereas the oxygen consumption of the Brown-Pearce epithelioma is stimulated in hypoglycemic sera, its aerobic glycolysis is halved.

Within the past few years, observations have indicated a close relationship between the rate of glycolysis (whether aerobic or anaerobic) and the pH of the medium. Schrek (10), studying the survival of many kinds of tissue cells from different species when exposed to a glucose-containing salt solution kept at 45° C., found that all kinds, whether exposed to oxygen or not, produced acid, and that the increase in acid production with time

TABLE 1

THE AEROBIC LACTIC ACID PRODUCTION IN NORMAL AND IN SHOCK SERA EXPRESSED IN MG/MG OF DRY TISSUE*

Exp. no.	Lactic acid production in normal serum (mg.)	Glucose level (mg. per cent)	Lactic acid production in shock serum (mg.)	Glucose level (mg. per cent)
1	0.036	140	0.023	35
2	0.036	155	0.010	26
3	0.025	144	0.018	33
4	0.042		0.017	44
5	0.021	122	0.014	
AV.	0.032		AV. 0.016	

* Over a period of approximately 2 hours, 45 minutes at room temperature and 75 minutes at 37.5° C.

paralleled the decrease in pH. Summerson, Gilder, and Lee (12) reported that the aerobic production of lactic acid by lymphosarcoma cells proved to be a linear function of the pH. (For example, 1.05 μl lactic acid/10^6 cells/hr at pH 7.6; 0.78 μl at 7.4, and 0.3 μl at 6.9.) The only kind of acid produced by lymphosarcoma, they maintained, is lactic, and as glucose disappears an approximately equivalent amount of lactic acid appears. LePage (8), using homogenates of rat tissues, has demonstrated that the rates of glycolysis (anaerobic) of normal tissues are equal to or greater than that of rat carcinoma (Flexner-Jobling).

These observations appear to accentuate the importance of knowing the relationship of glycolysis to respiration.

The Warburg ratio.—In his book on tumor metabolism Warburg (14) evolved a formula based on theoretical considerations for estimating by the manometric method the ratio of the total sugar decomposed (glycolyzed) to the quantity of sugar oxidized. In this paper this ratio is designated as Warburg's ratio. Since 1 mole of lactic acid will release 1 mole of CO_2 from a bicarbonate medium, and since 1 mole of lactic acid is equivalent to $\frac{1}{2}$ mole of glucose, the $Q_L^{O_2}$ value of a tissue expressed in moles divided by 2 will give the moles of glucose glycolyzed. Assuming that all the oxygen consumed by the tissue goes to burn glucose (probably an unwarranted assumption), the Q_{O_2} value expressed in moles divided by 6 will give the moles of glucose oxidized. The ratio of the $Q_L^{O_2}$ value to the Q_{O_2} value multiplied by 3 will then be equivalent to the ratio of the quantity of glucose glycolyzed to the quantity of glucose oxidized.

In studying the Flexner-Jobling rat carcinoma, Warburg found a value of 12 for his ratio, which he interpreted as meaning that for every 13 molecules of glucose attacked 12 were split into lactic acid. On this basis he states that the metabolism of the carcinoma tissue in oxygen is predominately one of glycolysis.

In summarizing certain of the results recorded in the literature, Burk (3) lists values for Warburg's ratio (aerobic glycolysis to equivalent respiration) ranging from 5.1 for human skin carcinoma to 17.7 for carcinoma of the penis, with intermediate values of 10 and 12 for the Flexner-Jobling rat carcinoma and the Rous chicken sarcoma, respectively. Inasmuch as these values are based on manometric measurements, it seemed to us important to determine Warburg's ratio for the Brown-Pearce epithelioma more directly by chemical methods.

Aerobic glycolytic studies were made in normal serum under approximately 700 mm. partial pressure of oxygen, and the lactic acid produced and the glucose consumed were ascertained as described in the section on methods. Lactic acid and glucose levels in the normal serum were determined; then levels of these substances were ascertained for the serum after the tumor had respired in it for approximately 2 hours, 45 minutes at room temperature and 75 minutes at 37.5° C. The differences represented the lactic acid produced and the glucose consumed. Attempts were made by careful observation to keep the quantities of tissue in each flask as nearly the same as possible. The ratios determined for lactic acid production to glucose consumption are shown in Table 2.

The consistency of the results may be attributed to the following features of the experiment:

1. The time variable was eliminated.
2. Errors in sampling were reduced, as the lactic acid and sugar determinations were made on the same zinc filtrate.
3. The necessity for tissue controls was abolished.

4. The ratio obtained did not depend upon recovery and weighing of tissues.

The results show that 63 per cent of the glucose metabolized appears as lactic acid. In other words, for every 100 mg. of glucose metabolized, 63 mg. are glycolyzed and 37 mg. oxidized or otherwise destroyed.

Assuming that all the glucose which is not glycolyzed is completely burned, calculating Warburg's ratio from Table 2, a value of 63/37 or 1.7 is obtained. This value is considerably lower than those listed by Burk, based on manometric measurements, for other malignant tissues, and corresponds more nearly to the values he lists for benign tumors. However, if the data obtained in this investigation are calculated by the method of Warburg, values for his ratio of the same order as those listed by Burk for malignant tissues will be obtained. Table 3 summarizes the results of experiments conducted in normal serum in which tissue controls were used so that $Q_L^{O_2}$ values could be determined. It is evident that the ratio of lactic acid produced to glucose consumed should be employed rather than manometric measurements as a more accurate index of the carbohydrate metabolism of a tumor.

SUMMARY

1. A study was made to determine the extent to which the metabolism of the Brown-Pearce tumor could be altered by lowering the circulating glucose level with insulin. The metabolism of tumor slices was investigated in normal sera from rabbits which had been on a fast and in sera prepared from animals in insulin shock. Oxygen consumption was measured manometrically and aerobic lactic acid production determined colorimetrically.

2. Six experiments were conducted in which the glucose level of the shock sera was restored approximately to normal or above, and six in which the effect of the addition of glucose to normal serum was ascertained. A graph showing the relationship between serum glucose and Q_{O_2} values was determined.

3. The ratio of aerobic lactic acid production to glucose consumption in normal serum was ascertained. The ratio of aerobic glycolysis to equivalent respiration was calculated by data determined chemically and data determined manometrically.

4. The oxygen consumption of Brown-Pearce tumor slices is increased by 17.2 per cent and the aerobic lactic acid production decreased to 50 per cent in sera prepared from animals in insulin shock, as contrasted to determinations made in sera taken from the same animals on fast for 14–24 hours.

5. The stimulating effect of hypoglycemic sera on oxygen consumption is destroyed by the addition of glucose.

6. A value of 0.63 was obtained for the ratio of aerobic lactic acid production to glucose consumption in normal sera.

TABLE 2

THE RATIO OF AEROBIC LACTIC ACID PRODUCTION TO GLUCOSE CONSUMPTION IN NORMAL RABBIT SERUM

Exp. no.	Lactic acid/mg (mg.)	Av.	Total lactic acid production (mg.)	Total glucose consumption (mg.)	Lactic acid/glucose	Av.
1	0.032		0.610	1.13	0.54	
	0.044		0.637	0.89	0.72	
	0.033	0.036	0.487	0.82	0.59	0.62
2	0.037		0.650	0.93	0.70	
	0.038		0.691	1.17	0.59	
	0.033	0.036	0.650	1.02	0.64	0.64
3	0.032		0.735	1.07	0.69	
	0.022	0.027	0.435	0.82	0.53	0.61
4	0.061		0.788	1.19	0.66	
	0.051		1.032	1.81	0.57	
	0.054	0.055	0.598	1.00	0.60	0.61
5	0.047		0.938	1.42	0.66	
	0.043		0.938	1.49	0.63	
	0.048	0.046	0.694	0.99	0.70	0.66
6	0.034		0.594	1.01	0.59	
	0.042	0.038	0.748	1.19	0.63	0.61
					Av.	0.63

TABLE 3

DETERMINATION OF WARBURG'S RATIO IN NORMAL SERUM (By Manometric Method)

Exp. no.	Lactic acid/mg ht	$Q_L^{O_2}$	Q_{O_2}	$3Q_L^{O_2}/Q_{O_2}$
1	0.024	5.95	3.38	5.3
2	0.017	4.22	2.89	4.4
3	0.035	8.68	3.61	7.2
4	0.050	12.40	4.72	7.9
			Av.	6.2

7. A comparison between the metabolism of tumors taken from rabbits on fast with that from those in insulin shock was made in CO_2-free Ringer's phosphate medium. No significant difference in Q_{O_2} and R.Q. values was found.

8. In the light of determinations by chemical methods, it appears that the Warburg ratio ascertained manometrically does not accurately reflect the extent of tumor glycolysis. It is concluded that the ratio of lactic acid produced to glucose consumed should be employed as a more precise index of carbohydrate metabolism.

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The Development of Resistance to Reinoculation and of Circulating Cytotoxins in Response to Heterologous Ocular Tumor Transplantation in the Guinea Pig*

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The superiority of the eye as a site for the transplantation of cancers and other tissues might be due to differences in the development of immunological events in this area as compared to other transplantation sites. Investigation of this hypothesis is stimulated by two considerations. First, there is increasing evidence that immunological factors determine the fate of many homografts and heterografts (5, 6, 7, 14). Secondly, there is evidence that immune reactions on the part of the eye are, in a few circumstances, peculiar in rate and character. For example, some of the peculiarities of the interstitial keratitis of congenital syphilis have been attributed to a relative failure of the cornea to participate in immunological developments occurring elsewhere in the infected individual (16). The fact that the eye contains two organ-specific substances, lens protein and uveal pigment (15), suggests the possibility of a compensatory local suppression or block in the mechanism of foreign protein reactions. In view of these considerations, it appeared worth while to attempt to determine if a delay or failure in the occurrence of immunological reactions could be responsible for the superiority of the eye as a transplantation site.

Immunity to grafts of homologous adult tissue transplanted in the eye, and subcutaneously, has been demonstrated by Woodruff and Woodruff (14). These authors studied the relative immunizing capacity of these two routes for homografts of thyroid tissue in thyroidectomized guinea pigs. They observed that both intraocular and subcutaneous grafts produced a state of relative resistance to the establishment of second grafts of the same tissue; immunizing grafts in either site produced a resistant state toward future grafts in both sites.

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Medawar (8) observed immunity of the eye to homologous grafted skin only when skin grafts in the anterior chamber were vascularized; nonvascularized grafts of skin in the immune eye behaved no differently from those in normal control eyes.

Greene (4) investigated the possibility that tumor transplants in the anterior chamber might be segregated from tumor anti-substances in the circulation by the action of the blood-aqueous barrier. He studied homologous transplants of various rabbit tumors and observed that, following growth of the tumor in one eye, both the testicle and the anterior chamber of the opposite eye were resistant to reinoculation, indicating a free passage into the eye of the factors responsible for the immunity.

The participation of the eye in immunity against the Brown-Pearce carcinoma in rabbits was studied by several observers, with conflicting results. Besredka and Bardach (1) observed that the anterior chamber participated in the generalized resistance to reinoculation on the part of rabbits immunized against this tumor. Saphir, Appel, and Straus (9), on the other hand, successfully grafted the Brown-Pearce carcinoma into the eyes of tumor-immune rabbits. Cheever and Morgan (2) found a relative but not absolute immunity under the same circumstances. These observers found that a monocular transplant failed to confer immunity on the opposite eye.

Resistance to reinoculation after heterologous ocular tumor transplants was observed by Schilling and Snell (10) with the mouse carcinoma MT8. The resistant state was found in both eyes after a monocular transplant.

In general, most authors agree that transplantation of alien tissue within the eye is followed by immune reactions, as is transplantation in other areas. The rate of development of immune reactions following ocular grafts, as compared with grafts elsewhere, has not been closely studied; it

is conceivable that this rate is of critical importance in the superiority of the eye as a transplantation site.

METHODS AND MATERIALS

The experiments reported herein were designed to test the development of immune reactions in response to transplantation within the eye, as compared to subcutaneous transplantation of tumors of alien species. Evidence for the occurrence of immune reactions following such transplantations was of two kinds: (*a*) the development of a refractory state, in which an initial transplantation was followed by failure of the same tumor to "take" when transplanted a second time to the same animal; (*b*) the development of circulating anti-tumor substances having a lytic effect on tumor cells growing in tissue culture.

Two different transplantable neoplasms were studied. One of these was a human breast carcinoma, which had been removed from an intracranial metastasis (13). This tumor had been transplanted to the anterior chambers of guinea pig eyes and maintained for 22 months by serial transplantations. The other tumor, more extensively studied, was the mouse carcinoma MT8 (10) carried in C3H mice. Guinea pigs were used throughout as the secondary or heterologous hosts.

The technics of the preparation of tumor samples and their transfer to the anterior chamber have been described (10, 11). Subcutaneous transplants were made with the same trocars used for the anterior chamber transplants, so that the amount of tumor introduced and the degree of maceration obtained were similar. Subcutaneous transplantations in the guinea pig were made principally with the mouse carcinoma. This tumor, when so transplanted, did not grow progressively. A severe inflammatory reaction developed about these transplants within 48 hours and subsided after 10–14 days.

The usual criteria (10) for the definition of growth of a tumor in the anterior chamber were applied: the transplanted fragment must vascularize and enlarge at least threefold, and histological identification must confirm the existence of growth of the tumor, at least in instances where the gross appearance of events in the eye left any doubt.

The tissues to be cultured *in vitro* were placed on cover slips where they were allowed to dry momentarily. This produced adhesion to the cover slip without the addition of other material. The cells were cultured at 37.5° C. in a mixture consisting of 4 parts of Fischer V-614 synthetic medium (3) and 1 part of Simms's ultrafiltrate of ox blood (12). A small particle of dried chick embryo extract was added. Each flask contained five to eight pieces of tissues and 1.0–1.5 cc. of medium. The aqueous humor or serum to be tested was added to this medium. About three-fourths of the explanted specimens grew actively; those flasks with poor tumor cell growth were discarded.

EXPERIMENTAL

The refractory state.—Previous work (10) had demonstrated that transplantation of MT8 to the guinea pig eye produced, after regression, a marked decrease in the survival of second ocular transplantations of portions of the same tumor. Transplantation into one eye conferred the refractory state on both eyes. The refractory state developed whether or not the tumor "took" and grew on the initial transplantation. It remained to be determined if subcutaneous transplantation also produced the refractory state to intraocular transplantation. Accordingly, seventeen guinea pigs (in four groups) received subcutaneous transplants of MT8. Seven to 12 days later new specimens of the same tumor freshly removed from other mice were transferred to both eyes. No growth of the tumor occurred in any of the 34 eyes. The expected incidence of growth of this tumor in the eyes of normal guinea pigs is about 50 per cent (10, 13).

It was desired to determine if transplantation via the ocular route produced the refractory state as quickly as was the case with the subcutaneous route. Four groups of guinea pigs, 38 animals in all, received monocular transplants of MT8, made in the right eye. Seven to 12 days later each animal save one received a new transplant of the same tumor in the opposite (left) eye. Growth of the transplants occurred in the right eyes in 24 animals, and in the secondly transplanted left eyes in four animals.

Previous work (13) had shown that it is occasionally possible to obtain growth of heterologous tumors transplanted within the stroma of the corneas of guinea pig eyes. The following experiment was done to determine whether the refractory state was exhibited by the cornea as well as by the anterior chamber. A series of fifteen guinea pigs was rendered "immune" by bilateral ocular transplantation of MT8 into a group of four animals, and by subcutaneous transplantation into eleven animals in two groups. After regression of the intraocular tumor and absorption of the subcutaneous tumor, new transplants of MT8 were made into 27 of the corneas of the above animals. Following this procedure, the corneas usually became vascularized, but the transplants usually did not. In 22 cases the transplant was absorbed without becoming vascularized. In the last group

of six animals, however, five of the twelve intracorneal transplants did become vascularized as in a successful "take," but regression followed almost immediately, and the transplant failed to enlarge threefold. Four corneas in two normal animals received transplants of MT8 as simultaneous controls for this group, and typical growth with marked enlargement occurred in three. In an earlier series (13) the incidence of "takes" in the cornea was 69 per cent for this tumor. Evidently the cornea, as well as the anterior chamber, participates in the refractory state.

In summary, the above data (Table 1) indicate that subcutaneous and ocular heterotransplantation of the mouse tumor MT8 produce a state refractory to the establishment of the same tumor on ocular retransplantation; the refractory state develops within 7 days after either subcutaneous or ocular transplantation.

TABLE 1

RESULTS OF INTRAOCULAR TRANSPLANTATION OF MT8 WHEN PRECEDED BY TRANSPLANTATION TO THE OPPOSITE EYE OR TO THE SKIN

1st transplantation site	No. eyes with tumor growth, 1st site	Interval between 1st and 2d transplantations (days)	2d transplantation site	No. eyes with tumor growth, 2d site
Skin, 2 animals		7	Right and left anterior chamber, 4 eyes	0
Skin, 10 animals		8	" " " " " 20 "	0
Skin, 2 animals		9	" " " " " 4 "	0
Skin, 3 animals		12	" " " " " 6 "	0
Right anterior chamber, 9 eyes	6	7	Left anterior chamber, 9 eyes	1
" " " 9 "	6	8	" " " 8 "	1
" " " 11 "	5	9	" " " 11 "	2
" " " 9 "	8	12	" " " 9 "	0

Tissue culture.—Aqueous humor, as well as blood serum, of guinea pigs that had received tumor transplants exhibited a characteristic lytic effect on the cells of the same tumor growing in tissue culture. The general character and course of this cytotoxic effect, as observed microscopically, was as follows:

In the affected cells, large numbers of cytoplasmic droplets or granules appeared. The cell walls became crenated, and the characteristic short processes disappeared. The cells often became noticeably swollen and rounded. Many cells disintegrated entirely, leaving clumps of granules (Fig. 1). Some of the granules adhered to the cover slip, and even after repeated washings and reculturing they remained unchanged. Many cells were seen to float off the cover slip and were washed away. The main mass of the explant became smaller and broken. Finally, growth was reestablished in some flasks after washing and the addition of fresh normal medium (Fig. 2).

The effects of the anti-substance on growing tumor cells were usually vigorous and dramatic and appeared within 8–24 hours. On the other hand, changes in pH or in temperature, or the presence of bacterial contamination or several months' aging did not produce nearly so rapid or so extensive effects. The human carcinoma, the mouse tumor MT8, human fibroblasts, mouse fibroblasts, and chick embryo heart could be grown in tissue culture equally well when the usual media were replaced in whole or in part with the aqueous humor of normal horse, beef, or guinea pig eyes.

Five tissue culture flasks containing actively growing cells of the human breast carcinoma explanted from the guinea pig eye were treated by adding 1 or 2 drops of aqueous from guinea pig eyes in which other specimens of the same tumor had grown and had regressed 4–12 weeks previously. In every flask so treated the growing cells were injured in the manner described above.

Portions of the aqueous humor exhibiting the anti-tumor effect against the human mammary carcinoma did not alter cultures of human testis, chick embryo heart, MT8 tumor, and mouse and rat fibroblasts.

One flask in which the anti-tumor effect of "immune" aqueous humor had occurred was washed with standard media and further incubated. A few of the less affected cells appeared to recover, and multiplication was resumed. The anti-tumor effect occurred again when "immune" aqueous humor was now added a second time.

Similar procedures were carried out with the mouse tumor MT8. Ten flasks in which this tumor was growing in standard media were inoculated with 1 or 2 drops of aqueous humor from guinea pig eyes in which MT8 had been transplanted 4–12 weeks earlier and had grown and regressed. The tumor cells growing in tissue culture again were profoundly altered by such material. The cells became granular, shrank, and often disintegrated within 8–48 hours after the aqueous humor was added. In many of these flasks, multiplying tu-

mor cells were intermixed with actively growing fibroblasts, presumably mouse connective tissue explanted along with the tumor. These fibroblasts were not affected by the aqueous humor which destroyed the neighboring tumor cells. Aqueous humor exhibiting the *in vitro* effect against the mouse tumor did not affect cultures of the human breast carcinoma, embryonic lung epithelium of the mouse, or adult mouse or rat fibroblasts. MT8 explants were not altered by normal guinea pig aqueous humor or by aqueous humor exhibiting an effect against the human breast carcinoma. Aqueous humor obtained from either eye was effective, regardless of which eye had harbored the tumor.

In a second series, blood was withdrawn from the hearts of guinea pigs in which ocular growth and regression of MT8 had occurred. The blood was centrifuged, and 1 or 2 drops of the serum was added to each of five flasks of growing MT8 tumor cells. The tumor cells so treated were destroyed in each flask in the same manner as previously. Portions of the same serum did not affect mouse embryo lung epithelium in the one flask so tested, nor did it affect mouse fibroblasts in seven of the eight flasks tested. A sample of serum containing considerable hemolysed blood suppressed the growth of fibroblasts explanted from adult mouse connective tissue in one flask.

In a third experiment, three guinea pigs received subcutaneous transplants of MT8. Seven days later, aqueous humor was withdrawn from the eyes of these animals and added to six flasks in which MT8 tumor cells were actively growing in tissue culture. Again, the anti-tumor cell effect was observed in all flasks, most of the cells disintegrating within 48 hours.

In a fourth experiment, an additional eleven flasks of actively growing MT8 tumor cells received 1 or 2 drops of aqueous humor removed from guinea pigs which had received ocular transplants of MT8 7 days earlier. The anti-tumor effect *in vitro* of this aqueous humor was already present, as evidenced by the fact that most or all the cells in each flask were destroyed by the aqueous humor. In several of these eyes the tumor continued to grow. Two flasks were treated with aqueous humor from an eye with the anterior chamber half full of growing tumor. This material also exhibited the anti-tumor effect *in vitro*.

The data obtained by the tissue culture experiments are given in Table 2. In summary, a fairly specific *in vitro* anti-tumor cell effect was produced either by subcutaneous or by intraocular transplantation of the tumors investigated, and with the mouse tumor the effect was present at 7 days, regardless of which transplantation site was used.

TABLE 2

THE CYTOTOXIC EFFECT *in Vitro* EXHIBITED BY AQUEOUS HUMOR OR BY BLOOD SERUM FOLLOWING TRANSPLANTATION OF MT8 IN THE ANTERIOR CHAMBER OR SUBCUTANEOUSLY

Transplantation site	Interval between transplantation and removal of aqueous humor or of blood serum	No. of flasks with growing tumor tested for cytotoxic effect	No. of flasks with growing tumor in which cytotoxic effect occurred
Anterior chamber	4–12 weeks; aqueous humor	10	10
Anterior chamber	4–12 weeks; blood serum	5	5
Skin	7 days; aqueous humor	6	6
Anterior chamber, right eye	7 days; aqueous humor right or left eye	11	11

DISCUSSION

The results of the experiments described in this report agree with the majority of recorded observations on this topic; the eye does participate in immunological reactions to transplanted alien tissues, in the sense both that transplants within the eye excite systemic immunity, and that transplants within the eye are exposed to and may react with circulating immune bodies. The evidence presented here indicates that, in the case of the tumor studied, systemic immune reactions have occurred by the seventh day in response to ocular as well as to subcutaneous transplants. This makes it unlikely that the superiority of the eye as a transplantation site, at least for this tumor, is due to a relative slowness in the development of anti-substances following transplantation in this area.

The hypothesis of an ocular immunological weakness, even though it is not in accord with the available evidence, is by no means ruled out as a possible explanation for the unusual success of ocular transplants. While it is known that immune reactions do occur following ocular transplants, and that they occur essentially as quickly in the case of the eye as in other areas, it is not known if these reactions occur to the same degree or as strongly for the ocular as for other transplantation routes; quantitative titrations are needed.

Moreover, only two immunological effects have been studied; it is not known if other kinds of immunological reactions occur following ocular tissue transplantation. Finally, it is not certain that the immunity which follows both ocular and subcutaneous transplantations is the critical event in determining the survival or destruction of an intraocular tumor transplant.

There is even some evidence that the reactions studied are not actually those which determine the fate of the original intraocular transplants. For example, the tumor cytotoxin so quickly effective against the tumor *in vitro* appears at or before the time the tumor is growing in the eye; as the tumor vascularizes, the local supply of circulating anti-substances must improve, yet this is the time of most vigorous tumor growth. The anti-substance is not being absorbed by the tumor faster than it can be delivered, because, as described above, the aqueous humor from an anterior chamber half-full of growing tumor still exhibited the cytotoxic effect against the tumor *in vitro*. The same apparent conflict applies in the case of the state of resistance to retransplantation; this resistance appears just at a time when the original transplant has gained a precarious vascular supply and is beginning to grow most actively. Woodruff and Woodruff (14) observed prolonged survival of vascularized homologous thyroid grafts in the eyes of thyroidectomized guinea pigs even when a high degree of immunity to the same tissue was evidenced by the behavior of secondary grafts made subcutaneously or into the opposite eye. Resistance appeared to be directed against the establishment of a new graft, while a vascularized and already established graft appeared to be protected and survived.

It is interesting that Medawar's observations (8) are directly at variance with those of Woodruff and Woodruff (14). Medawar, in studying the fate of homologous intraocular skin grafts in immunized rabbits, observed that those skin grafts that did not vascularize also escaped rapid destruction, and, conversely, that vascularized homologous skin grafts within the eyes of immunized rabbits were injured promptly and profoundly.

We have observed vascularization of the cornea in response to intracorneal transplants of MT8 in immune as well as in normal animals. This suggests that immunity does not depend upon a suppression of a vaso-trophic effect. The occurrence of corneal vascularization under these circumstances makes it impossible to reach any conclusions about the presence of immune factors in the undisturbed cornea.

The observed specificity of the cytotoxin for the particular tumor cells which provoked it is unusual. In the case of the mouse tumor, tumor cells were destroyed while fibroblasts from the mouse host, explanted along with the tumor, were not affected. There are two explanations for this considerable specificity. In the first place, this tumor excites a minimum of desmoplasia in the mouse and (apart from host vascular tissue) is almost a pure culture of tumor cells. The same is true of the human carcinomas growing in the guinea pig eye, in which no human vascular or connective tissue is represented. Secondly, the degree of specificity of tumor and other cytotoxins has been considered by Harris (5) to be a function of their concentration—specificity diminishing as the titer rises. In our experiments, the concentration of the cytotoxic agent must have been low, because frequently a few of the cells in a single flask were not destroyed by the toxin; presumably its concentration happened to be optimal for specificity.

SUMMARY

1. A state of resistance to the growth of an ocularly transplanted mouse tumor MT8 develops following either subcutaneous or intraocular transplantation of the same tumor in guinea pigs.

2. The resistant state to retransplantation in the eye is detectable at 7 days whether the original immunizing transplantation is made in the eye or subcutaneously.

3. Cytotoxins injurious to the cells of the same tumor growing *in vitro* are detectable at 7 days after transplantation either in the eye or subcutaneously; the cytotoxins are found in both eyes and in the blood plasma after a monocular or a subcutaneous tumor transplant.

4. It is concluded that the rate of development of these immunological reactions is not a critical factor in the superiority of the guinea pig eye as a transplantation site for these tumors.

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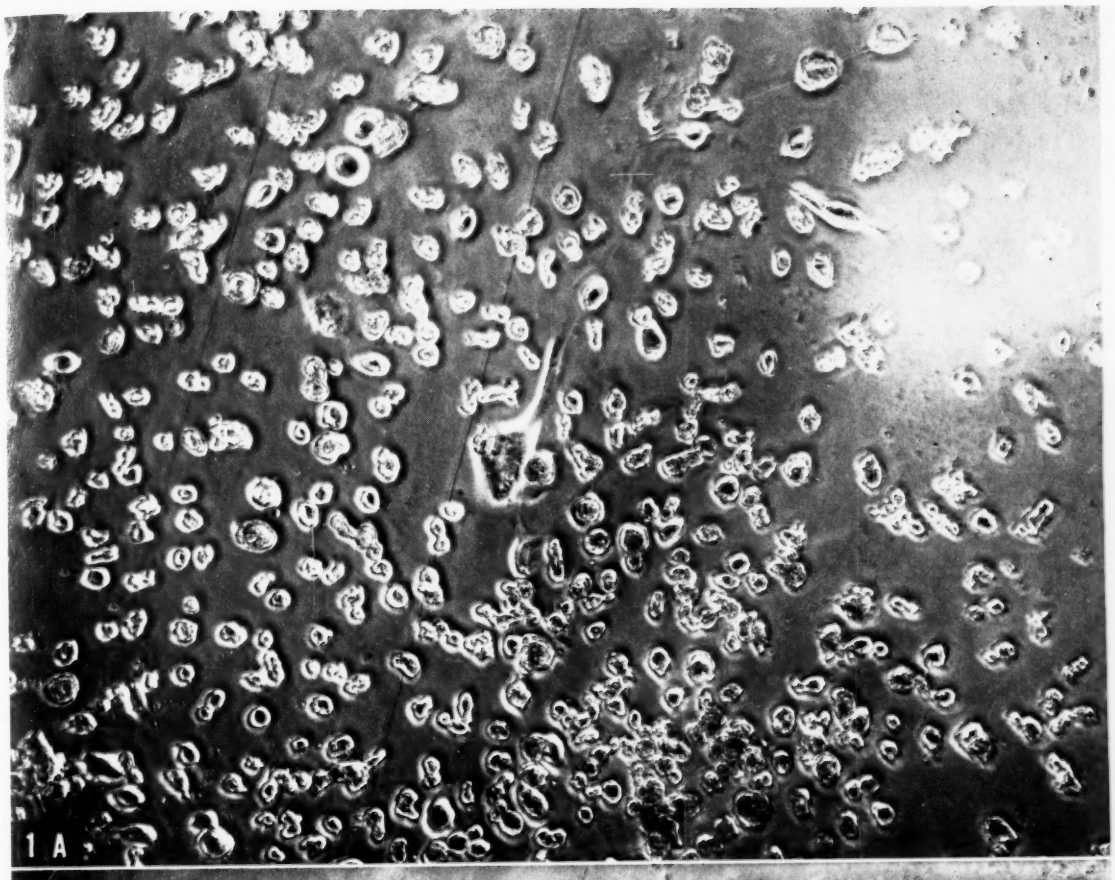
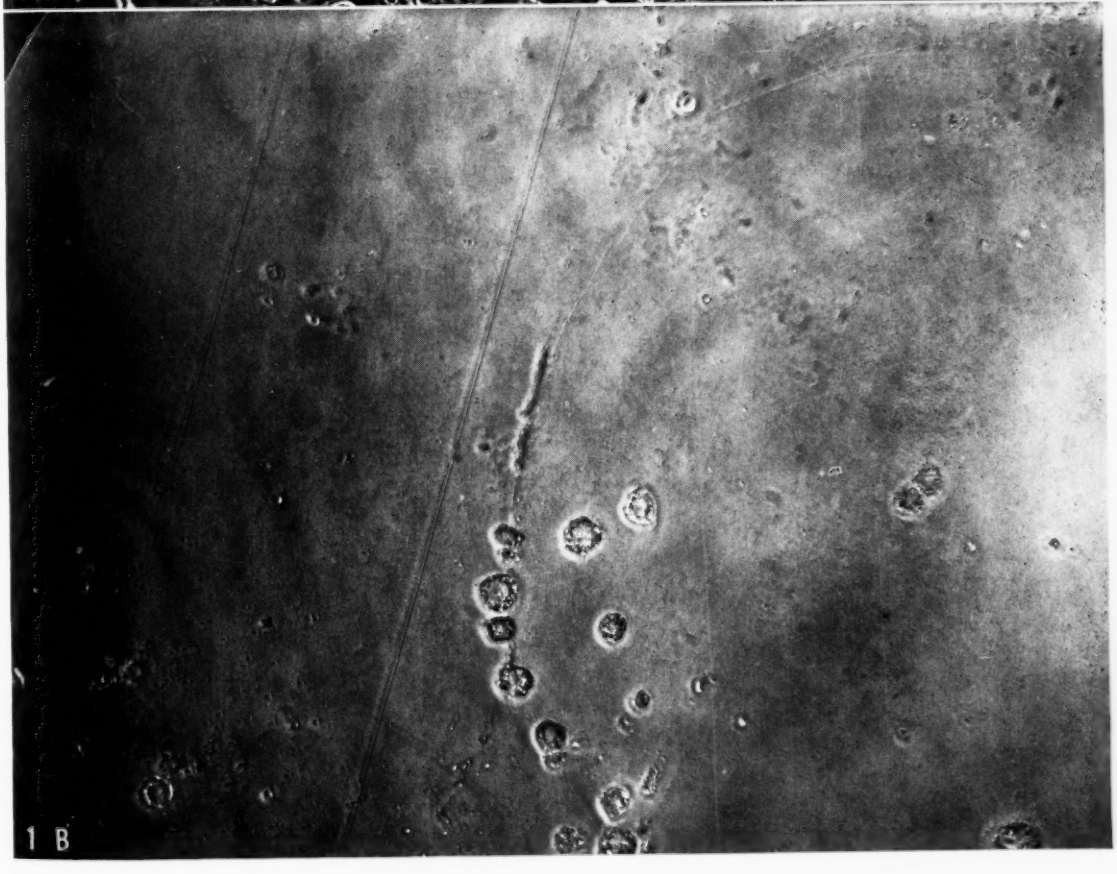
FIG. 1.—(*A*) Phase photomicrograph of MT8 tumor cells as cultured *in vitro* and (*B*) the same field 12 hours after the addition of aqueous humor from the eye of a guinea pig which had received an ocular transplant of MT8 2 weeks previously.

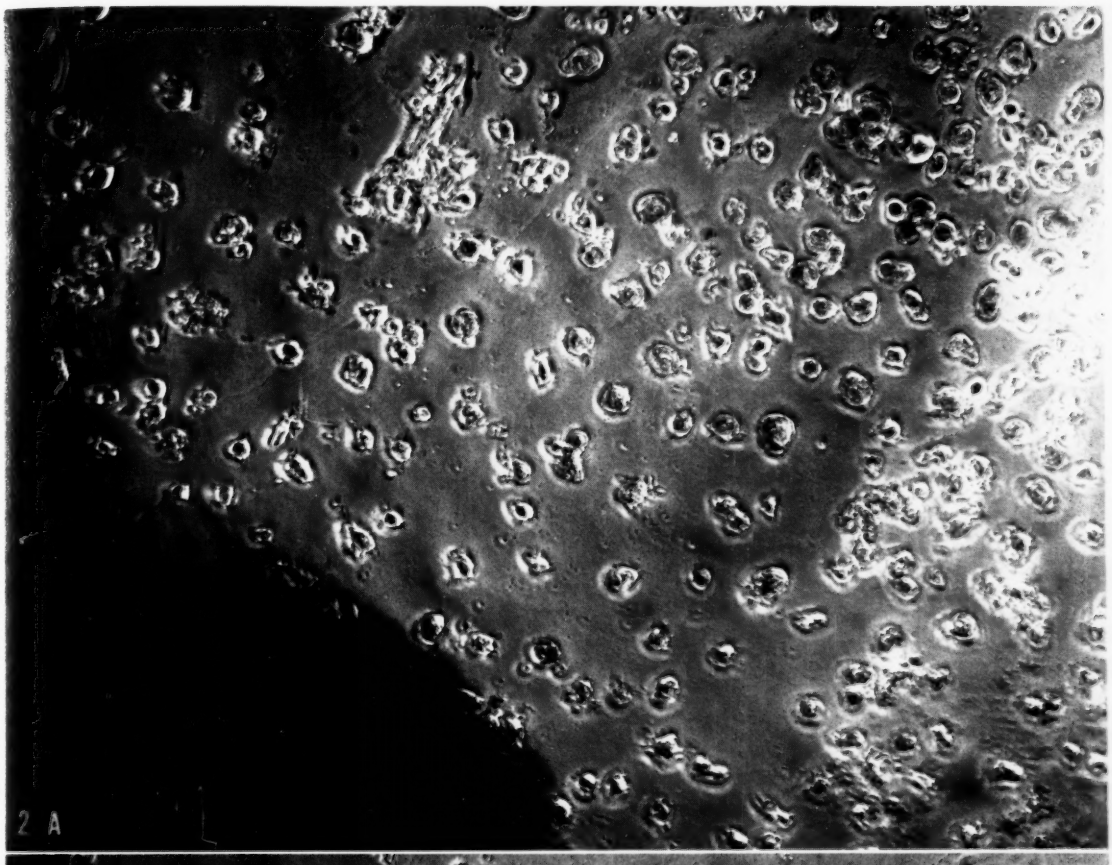


1 A

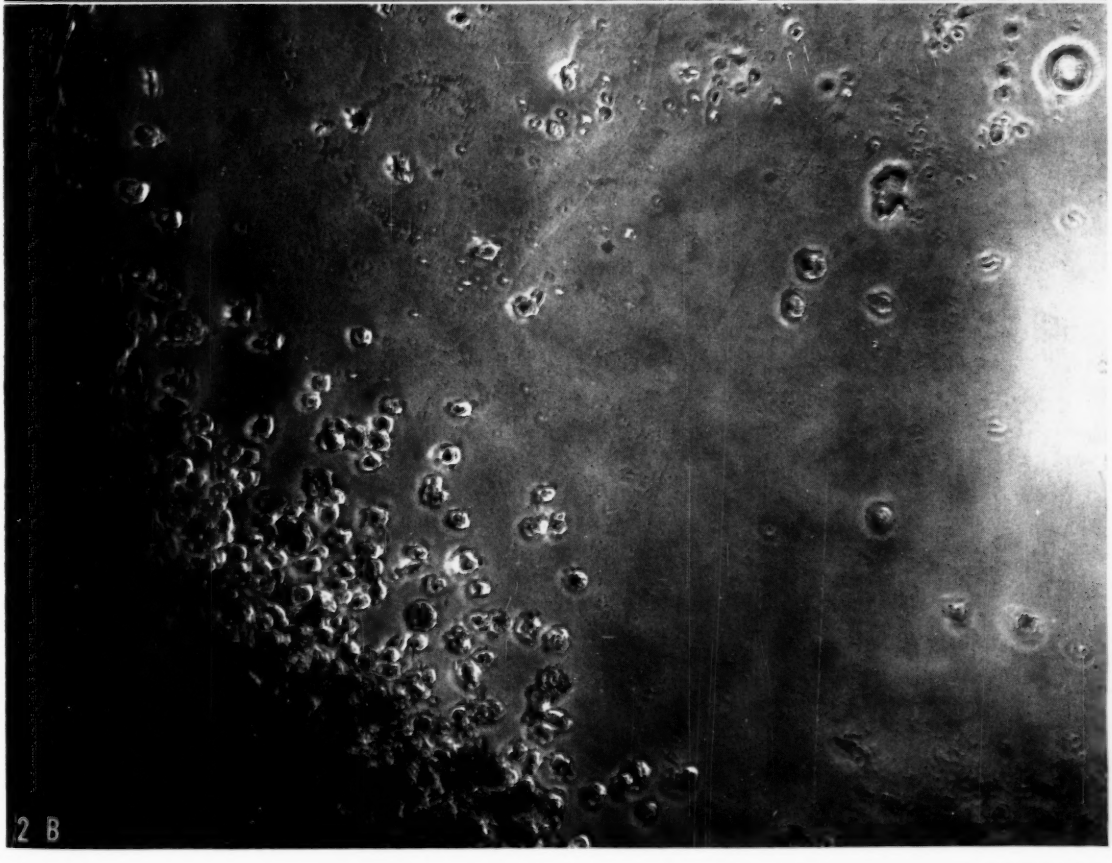


1 B

FIG. 2.—Phase photomicrograph of MT8 tumor cells (*A*) before and (*B*) 48 hours after the addition of aqueous humor from the eye of an immune animal, and 24 hours after washing and substitution with normal media. The original cells have disappeared and new cells have grown out from the margin of the explant.



2 A



2 B

Systemic Effects of Tumors in Rats*

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Systemic effects of tumors are regarded as the changes produced in tissues of the host which are remote from the tumor and in which no evidence of metastatic malignant cells is found. They may be anatomical, of a nature described by Parsons (17), or the type of biochemical change reviewed by Greenstein (11).

It has been suggested on histological grounds that the clinical state of malignant cachexia is due to hypofunction of the adrenal (6), and this view may be supported by certain clinical studies (18, 19, 34). In view of the number of metabolic factors known to be influenced by the adrenal cortex (30), the possibility that some systemic effects could be explained by a primary action on the pituitary-adrenal system was investigated.

Liver catalase activity was chosen for study as a well established systemic effect (1, 2, 7, 12), and adrenalectomy leads to a diminished activity of liver catalase (4). Hemoglobin and liver catalase have a common prosthetic group (29), and anemia has been related to tumor growth (28) and to the adrenal cortex (32).

The enlarged adrenal low in ascorbic acid and cholesterol which has been described in the tumor-bearing animal (22, 14) would be compatible with exhaustive hypofunction of the adrenal cortex (24), particularly in conjunction with the diminished liver glycogen deposition (35) and lymph node hypertrophy (15) which have been demonstrated in tumor-bearing mice.

It was decided to study these multiple systemic effects in a single animal at different stages of tumor growth, and the rat was chosen to provide the required amount of tissue. Thymus weight was followed as an example of lymph tissue, for it was assumed to react to experimental procedures in a manner similar to lymph nodes (31).

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METHODS

Young, male Sprague-Dawley (Holtzman) rats[1] were maintained on a diet of Purina Fox Chow and tap water in a room maintained between 72° and 78° F. The rats were bred in the laboratory or supplied by the Holtzman-Rolfsmeyer Company,[1] the latter rats being adapted to the animal room for at least 10 days before use. Bilateral grafting was done aseptically when the rats were approximately 6 weeks of age, a tumor suspension being used. The Walker 256 carcinoma was grafted either subcutaneously or intramuscularly, but only the latter method was used for the Jensen sarcoma. Normal male rats served as controls and were killed at the same time as tumor-bearing rats. Sixteen hours before sacrifice the rats were placed in clean cages without food but with water supplied *ad libitum*. At this time hemoglobin was determined on tail blood (8).

The rats received intraperitoneal injections of sodium pentobarbital in warm, normal saline at a dose level of 5 mg/100 gm body weight. Under anesthesia the abdomen was opened, the right adrenal transferred to formolsaline, and the left placed in a dish of ice-cold normal saline. A second operator removed a piece of liver for glycogen determination (9). This was rinsed in ice-cold saline, blotted dry, weighed, and introduced with minimum delay into a tube of hot 30 per cent potassium hydroxide. Meanwhile, the left adrenal had been freed of adherent fat, blotted dry, sectioned with a razor blade, and weighed on a torsion balance while wrapped in cellophane. One piece was introduced into trichloroacetic acid for estimation of ascorbic acid (21). The other half was homogenized with a loose pestle in a tube containing acetone, and an equal amount of absolute alcohol was added for the extraction and determination of cholesterol (27). The remainder of the liver was ground and extracted for the estimation of liver catalase activity (12) and the thymus weighed on a torsion balance. After 48 hours' fixation, the adrenals were washed in running tap water for an hour,

[1] Holtzman-Rolfsmeyer Co., Rt. 4, Madison 5, Wis.

sectioned on a freezing microtome, and stained with Sudan IV (5).

The body weights recorded are those prior to sacrifice and include both tumor and carcass weight. The tumors were measured in two diameters and are presented as the mean diameter of both measurements. Tumors were not weighed in all cases, but sufficient data are available to state that in the 20-mm. group they formed approximately 5 per cent of the body weight, in the 30-mm. group 15 per cent, and in the 40-mm. group 30 per cent of the body weight. With the subcutaneous grafts, the tumors attained the 10-, 20-, 30-, and 40-mm. diameters in 1, 2, 3, and 4 weeks, respectively. The intramuscular grafts grew at a somewhat faster rate.

TABLE 1

BODY WEIGHT OF CONTROL AND TUMOR-BEARING RATS

Tumor size	Body weight (gm.)
Control	170±4(7)
10 mm.	160±7(16)
20 mm.	161±7(10)
30 mm.	173±11(10)
40 mm.	182±12(10)

± Standard error of the mean. Number of observations in parentheses.

TABLE 2

EFFECT OF TUMORS ON ADRENAL AND THYMUS WEIGHT

Tumor size	Adrenal weight (mg.)	Thymus weight (mg.)
Control	15.4±0.8(7)	387±2.3(7)
10 mm.	17.5±0.4(16)*	420±35(12)
20 "	18.8±0.3(10)†	324±45(10)
30 "	21.3±2.0(10)*	288±86(5)
40 "	30.2±4.0(9)†	156±29(8)†

± Standard error of the mean. Number of observations in parentheses.
* $P < 0.05$ in t test.
† $P < 0.01$ in t test.

Values of P in the t test of Fisher are regarded as significant at the 0.05 level and highly significant at 0.01 (26).

RESULTS

The Walker and Jensen tumors gave comparable systemic effects, and the results obtained from rats bearing these tumors have been combined.

The mean body weights of the different groups are given in Table 1. There is no significant difference between the mean weights of the groups, and the adrenal and thymus weights are thus regarded as subject to valid comparison between groups.

Thymus and adrenal weights are presented in Table 2. There is a progressive increase in adrenal weight and a fall in thymus weight after the tumors have attained a size of 20 mm.

The results of the estimation of hemoglobln and liver catalase activity are tabulated in Table 3. Hemoglobin falls progressively from the 20-mm. tumor to attain a preagonal value of approximately 50 per cent. The loss of liver catalase activity is of the same order. The increase in catalase activity in rats bearing small tumors has been reported (13) and is significant in this series.

In Table 4 the effects on adrenal cholesterol and ascorbic acid have been recorded. The values for cholesterol and ascorbic acid fall to 60 per cent of the control at the 40-mm. stage.

TABLE 3

EFFECT OF TUMORS ON HEMOGLOBIN AND LIVER CATALASE ACTIVITY

Tumor size	Hemoglobin (gm/100 ml)	Liver catalase activity ($K \times 10^4$ at 0.1 mg N/ml)
Control	15.4±0.3(7)	1570±74(6)
10 mm.	16.2±0.8(16)	2079±132(16)*
20 "	11.8±1.2(10)†	1596±119(10)
30 "	10.7±1.1(10)*	1332±125(10)
40 "	8.6±0.5(10)*	753±81(10)*

± Standard error of the mean. Number of observations in parentheses.
* $P < 0.01$ in t test.
† $P < 0.05$ in t test.

TABLE 4

EFFECT OF TUMORS ON ADRENAL CHOLESTEROL AND ADRENAL ASCORBIC ACID

Tumor size	Adrenal cholesterol (mg/100 mg)	Adrenal ascorbic acid (mg/100 mg)
Control	4.68±0.52(7)	0.413±0.012(7)
10 mm.	4.16±0.38(16)	0.372±0.012(15)*
20 "	3.79±0.52(10)	0.324±0.017(10)†
30 "	3.25±0.45(9)	0.314±0.022(9)†
40 "	2.97±0.44(10)*	0.238±0.027(9)†

± Standard error of the mean. Number of observations in parentheses.
* $P < 0.05$ in t test.
† $P < 0.01$ in t test.

Histological examination revealed a loss of sudanophilia from the adrenals of the tumor-bearing rats. The loss of sudanophilia paralleled the loss of cholesterol from the adrenal.

DISCUSSION

The Walker 256 carcinoma at an early stage of growth reduces the food intake of the host, and as the tumor increases in size the carcass loses weight (16). The suggestion has been made that this may be a factor in the loss of liver catalase activity in the light of the known effects of starvation on liver catalase (7). Experiments have been reported which indicate that in the force-fed tumor-bearing

rat no loss of carcass weight occurs, but systemic effects are present (3). Thus, failure to record food consumption in this study does not render the results invalid.

The present experiments are not in agreement with the statement that tumors forming 5 per cent of the body weight cause a diminution in catalase activity (11) but do agree with observations that half the liver catalase activity is lost from animals bearing tumors of 15–30 per cent of the body weight (7). The catalase effect appears to be biphasic, as has been reported by Greenstein in rats (13) but not noted by Adams, who found an asymptotic relation between tumor weight and per cent inhibition of catalase in mice (1).

The earliest significant effect produced by a tumor in this investigation is the hypertrophy and loss of ascorbic acid from the adrenal at the 10-mm. stage, followed by anemia at the 20-mm. stage. That the degree of anemia is a consistent and reproducible finding in tumor-bearing rats is suggested by the fact that comparable groups of rats bearing the Walker carcinoma yielded hemoglobin values of 8.34 ± 0.49, 8.50 ± 0.40, and 8.03 ± 0.44 gm/100 ml at intervals of 6 months.

It was suggested that diminished liver catalase activity and anemia might be associated with hypofunction of the adrenal cortex in the tumor-bearing rat. Anemia has been produced by adrenalectomy in mice (32), and it has been demonstrated that adrenalectomy lowers liver catalase activity in the rat (4). But the diminution in catalase activity in the rat from which the adrenals are removed is only half as great as in the tumor-bearing animal. Thus, it is unlikely that hypofunction of the adrenal cortex is a major factor in the production of anemia and loss of liver catalase activity in the tumor-bearing rat.

The observed involution of the thymus in the tumor-bearing rat might be regarded as indicative of hyperfunction of the adrenal cortex (25) or inanition (20). Extrapolation to another species should be done with caution, but thymus involution and lymph node hyperplasia have been observed in tumor-bearing mice (15, 22) and appear to be independent of the pituitary (23). This throws some doubt on the interpretation of the present data, and further studies should be done to investigate the response of lymph nodes as well as the thymus to the presence of a tumor in the rat, and the role of the adrenal (33).

The results obtained from the study of adrenal weight, adrenal ascorbic acid and cholesterol, and sudanophilia might be interpreted as following the pattern of the stress reaction as exemplified by the Type III adrenal response of Sayers (24), on an extended time scale. This could be the result of an initial stimulation of the adrenal cortex being maintained and leading to eventual cortical exhaustion.

The progressive fall in adrenal cholesterol and ascorbic acid would suggest that the host was approaching the stage of adrenal cortical failure (24). In the absence of the determination of peripheral effects known to be influenced by the secretions of the adrenal cortex these changes in the adrenal are difficult to interpret. The determination of liver glycogen in rats starved for 16 hours gives such low results, and the data are so variable, that these experimental findings have not been presented. A plot of mean per cent change does indicate an initial increased storage of glycogen, with a diminution in the amount of liver glycogen in the terminal state. This problem should be studied with the use of intraperitoneal glucose (35).

It is probable that a state of adrenal cortical deficiency does occur in the tumor-bearing animal in the terminal stages of cancer, but further experimental substantiation is required. All the systemic effects cannot be explained on the basis of this deficiency, and some mechanism must be responsible for the many factors known to be altered in the tumor-bearing host.

As the result of the present and related investigations it is necessary to explain how the presence of a tumor at a remote site brings about a stimulation of the adrenal cortex, a diminution in the amount and activity of hemeproteins, and an involution of the thymus. Neither hormonal nor nutritional explanations seem to be adequate.

In his original studies on liver catalase activity in tumor-bearing rats, Greenstein suggested that "the effect of the transplanted tumor on the liver catalase is elicited by a toxic substance produced in the tumor and carried by the blood to the liver" (13). A recent abstract reports that such a substance can be extracted from a tumor (10). Adams favors the release of some substance from a tumor as an explanation of the diminished liver catalase activity of mice (1). A consideration of the systemic effects of tumors may lead to the revival of the concept of a cancer toxin and stimulate further research along this line.

A claim for specificity in relation to malignant tumors cannot be made for the observed systemic effects, and no explanation is available to account for these characteristic effects in tissues remote from a tumor. At the same time, they are manifestations of the profound metabolic changes produced in the host, and, as such, it is felt that they play an important part in the fatal outcome of cancer.

SUMMARY

Tumor-bearing rats exhibit enlargement of the adrenal with loss of ascorbic acid and cholesterol, atrophy of the thymus, diminution in liver catalase activity, and progressive anemia.

The thesis that rats bearing large tumors are in a state of hypofunction of the adrenal cortex requires further substantiation, and in any event such a state would not explain the observed systemic effects.

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Histochemical and Microchemical Changes in Experimental Cirrhosis and Hepatoma Formation in Mice by Carbon Tetrachloride*†

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Cirrhosis and hepatomas have been produced in experimental animals by numerous methods (7, 17, 19, 26, 31, 45). Although carbon tetrachloride has been frequently used for these purposes (5, 6), relatively few histochemical studies have been made. This paper will report a coordinated cytologic, histochemical, and biochemical study of mouse liver at intervals following repeated feedings with carbon tetrachloride.

Histochemical observations were made on nucleic acids, alkaline phosphatase, esterase, glycogen, and lipids. Quantitative chemical measurements were made for each of these constituents except glycogen. The chemical study was extended to include analyses for succinoxidase and acid phosphatase. In addition to these measurements, the tissues were chemically fractionated into acid-soluble, alcohol-ether-soluble, hot trichloroacetic acid-soluble, and residual protein fractions for the purpose of investigating possible alterations in the distribution of nitrogen and phosphorus within these fractions. Because of the insignificant alterations, these fractionation results for the most part will be excluded. The interpretation of chemical results was facilitated by quantitative cytologic measurements of cell and tissue constituents. The findings in hepatomas were compared to those in tissues at intervals after repeated carbon tetrachloride feedings. Similar studies have been made in mice at intervals after a single feeding of carbon tetrachloride (38, 42, 43), and investigations are in progress on other phases of normal, abnormal, and neoplastic liver growth in mice.

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MATERIALS AND METHODS

The procedures in these experiments were essentially the same as those employed in other studies (38, 42, 43). The animals were strain A male mice, 3 months of age. They were fed Purina Laboratory Chow throughout the experiment. Nine groups of animals were started at approximately monthly intervals. Twice weekly, 0.1 cc. of 40 per cent carbon tetrachloride in olive oil was injected directly into the esophagus by a specially designed trocar (25). A few animals died during the course of feeding. To avoid studying the effects of the immediate necrotic action of carbon tetrachloride, the treatment was stopped 14 days before the animals were killed. Both experimental and control mice were put on a fast 18 hours before sacrifice.

Mice were killed by cervical dislocation. Liver tissue was fixed in absolute alcohol and stained for alkaline phosphatase (8) and glycogen (10). Blocks were fixed in chilled acetone for the esterase technic (38), and in Stieve fluid (36) for Feulgen reaction for desoxypentosenucleic acid (35) and Heidenhain's connective tissue strain (23). Pyronin-methyl green staining (32, 40), and ribonuclease digestion (39) were used for studying ribonucleic acid, and hematoxylin and eosin for general observations. Liver fixed in 10 per cent neutral formaldehyde was stained for lipids with oil red O.

The chemical studies were carried out on livers from individual animals. Most of the measurements on hepatomas were made on single tumors and were compared with adjacent, non-neoplastic cirrhotic tissue from which nodules had been removed. Histochemical observations on tissues from many mice had been made before the chemical studies were started on the final series of mice.

Tissues for chemical studies were taken promptly after sacrifice. Hepatomas showing appreciable amounts of necrosis or inflammatory cells were not included in the experiments. All chemical analyses were made according to procedures previously described (42, 43) in conjunction with similar experiments on mice after a single feeding of carbon tetrachloride except for the phosphatase measurements, which were modified subsequent to this study (42). In the present investigation, acid phosphatase was determined at pH 4.5 instead of pH 5.5, as now being used. Both acid and alkaline phosphatases were determined at suboptimal levels of substrate concentration, which subsequently have been modified. However, it was felt that the methods utilized, although not representing optimal conditions for enzyme reaction, gave results which can be used for comparative purposes.

Data were calculated on a variety of bases, including wet weight, dry weight, dry weight minus lipid, and nitrogen. It was not possible to measure the content of moisture and lipid within some of the samples because of insufficient tissue—some of the fresh tumor samples weighing less than 150 mg. In Table 1 most of the data are presented on the basis of total tissue nitrogen. The changes on this basis were similar to those calculated by use of the other base lines. It is impractical to present the results of all samples measured, but some of the variation is indicated in Table 1.

TABLE 1

CHEMICAL STUDIES OF HEPATIC TISSUE

Mouse no.	Weeks on CCl_4	Tissue type	Mg N/gm wet wt	Mg P / Mg N	Mg DNA / Mg N	Mg PNA / Mg N	Gm total lipids / gm wet wt	Alkaline phosphatase*	Acid phosphatase†	Liver esterase‡	Succinoxidase§
1–8	0	A	36.3	.100	.087	.229	.141	.071	.171	154	677
			(31.9–40.0)	(.096–.103)	(.069–.113)	(.210–.251)	(.047–.239)	(.059–.102)	(.094–.237)	(105–245)	(575–845)
9–13	3	B	37.6	.100	.074	.225	.058	.062	.222	171	712
			(36.0–38.9)	(.097–.105)	(.067–.083)	(.213–.230)	(.051–.072)	(.051–.067)	(.186–.283)	(155–212)	(595–759)
14–18	7	B	36.9	.100	.079	.212	.054	.068		147	661
			(35.6–39.5)	(.095–.107)	(.070–.100)	(.199–.228)	(.041–.074)	(.062–.078)		(107–193)	(615–718)
19–22	12	B	36.1	.103	.115	.236	.050	.061	.232	222	732
			(34.8–37.1)	(.100–.104)	(.112–.121)	(.232–.247)	(.046–.059)	(.056–.067)	(.212–.253)	(177–272)	(710–750)
23–27	16	B	36.7	.105	.121	.228	.078	.098	.117	122	760
			(35.8–37.6)	(.102–.110)	(.109–.140)	(.186–.247)	(.061–.101)	(.072–.148)	(.113–.123)	(73–155)	(704–867)
28	20	C	36.4	.125	.135	.223		.058	.260	101	742
28	20	D	36.7	.095	.117	.232		.111	.228	99	464
28	20	D	37.5	.104	.106	.243		.070	.290	130	825
29	20	C	40.0	.111	.103	.228	.065	.054	.150	133	765
29	20	D	36.3	.104	.107	.244	.034	.044	.208	202	860
30	20	C	39.8	.099	.094	.223	.043	.056	.236	171	661
30	20	D	34.4	.111	.097	.259		.089	.196	129	690
30	20	D	35.8	.104	.111	.246		.058	.230	202	845
31	20	D	36.5	.099	.071	.222		.058	.231	188	496
32	20	C	37.3	.098	.098	.207	.104	.061	.225	158	559
32	20	D	34.3	.102	.093	.216		.047	.210	220	642
33	20	C	34.2	.104	.090	.228	.060	.039	.197	222	514
33	20	D	28.2	.120	.057	.303	.060	.113	.153	199	284
34	20	C	36.1	.101	.066	.218	.044	.047	.154	160	736
34	20	D‖	32.6	.110	.054	.235		.044	.117	278	637
Av.		C	37.3	.106	.098	.221	.063	.053	.204	158	663
Av.		D	34.7	.105	.090	.244		.070	.207	183	638

* Results given as mg phosphorus liberated/mg tissue N/hour at pH 9.2 and 37° C. with shaking.

† Units as for alkaline phosphatase, except at pH 4.5.

‡ Expressed as 100 µl CO_2 liberated/hour/mg tissue N.

§ Expressed as µl O_2 consumed/hour/mg tissue N.

‖ Hepatoma specimen from mouse 34 represents pool of three individual small tumors.

Figures in parentheses represent range of individual determinations.

A = livers from control rats.

B = Nontumorous livers at intervals after feeding carbon tetrachloride.

C = Cirrhotic livers.

D = Hepatoma.

OBSERVATIONS

ANATOMICAL AND HISTOLOGICAL FINDINGS

The incidence of cirrhosis and hepatomas is shown in Table 2. Cirrhosis (Fig. 1) occurs in all mice treated for more than 6 weeks. Grossly, the lesion varies from a barely recognizable wrinkling on the posterior surface of the liver in early stages to an atrophic, rough nodular appearance in later stages. Microscopically, definite fibrosis and pseudo-lobules are seen with Heidenhain's stain. Although the fibrosis is related to the duration of treatment, its extent and also the degree of regenerative and necrotic changes show individual variations, especially in the early stages. The

fibrosis is generally more marked around the central and sub-lobular veins. In the early stages, large amounts of granular, yellowish-orange ceroid pigment are frequently found in the proliferating fibrous trabeculae. This pigment is characterized by its acid-fast reaction and is stained by fat stains, but does not dissolve in ordinary lipid solvents (21). Later, both the fibrosis and pigment formation are greatly increased. In the late stages fibrosis becomes more diffuse, occasionally spreads into the lobules and quite frequently involves the portal canals. Subsequently, more numerous smaller lobules are formed, some of which contain no cells but only calcified granules. The fatty metamorphosis is moderate in this series, whereas calcification is fairly frequent. Heterotopic bone formation (37) was noticed in several livers.

The gross and microscopic pictures of hepatomas are similar to those described by other investigators (5, 6). The nodules are grayish-white, elevated, and clearly demarcated. Some larger ones, especially those in the left lobe, may grow downward into the lower abdominal cavity, forming spherical pedunculated masses. There is little difference in the distribution of nodules in the three main lobes; however, the small caudate lobe is involved only occasionally.

Microscopically, these nodules usually consist of solid cords of large or small liver cells without the normal lobular pattern (Fig. 2). Frequently, the small hepatoma cells contain prominent nucleoli and show more cytoplasmic basophilia; the more common large hepatoma cells usually exhibit vacuolar or hydropic changes in their cytoplasm and less cytoplasmic basophilia. This decreased cytoplasmic basophilia may be related directly to the vacuolar or hydropic changes. Mitotic figures and bizarre nuclei are only slightly more common in hepatomas than in cirrhosis.

Almost all the hepatomas show little or no fibrosis, while the adjacent cirrhotic areas are markedly fibrotic. The frequently observed fibrous capsule serves as a useful criterion for demarcating the tumor and implies its benign character.

Occasional groups of cells in a typical acinous structure can be found in hepatomas (Fig. 3). The lining cells may be high columnar, but bile pigment is not found. Angiomatous growths were rarely seen. Extra-medullary myelopoiesis was also occasionally encountered in hepatoma nodules.

Eosinophilic spherical cytoplasmic inclusion bodies were observed in occasional hepatoma cells. These bodies were counterstained with fast green in Feulgen preparations.

Rarely, one or two spherical structures were noticed in the nuclei. These were pink in hematoxylin-eosin preparations, blue with Heidenhain's azocarmine stain, and almost colorless with both pyronin-methyl green and the Feulgen reaction. Sometimes they were surrounded by a delicate layer of chromatin material. These occurred in the control, cirrhotic, and hepatoma tissues. Olitsky and Casals (27) have reported similar inclusion bodies in so-called normal mice and thought they were related to infection. In the present series, there is insufficient evidence to draw any conclusions as to the nature of these bodies.

The phosphotungstic acid hematoxylin stains for mitochondria (22) on material fixed in formaldehyde and mordanted with ferric chloride reveal a slight reduction in the mitochondria of hepatoma cells as compared to nearby cirrhotic liver cells.

TABLE 2

CIRRHOSIS AND HEPATOMAS PRODUCED IN STRAIN A MICE AFTER BI-WEEKLY FEEDINGS OF CARBON TETRACHLORIDE

No. of animals	Weeks of treatment	No. with cirrhosis	No. with hepatoma
15	0	0	0
7	2	0	0
5	6	5	0
6	11	6	0
7	15	7	6
4	20	4	1
7	26	7	4
10	30	10	9
5	35	5	5
2	39	2	2

General Chemical Data

Some of the chemical findings are summarized in Table 1. The nitrogen and phosphorus content did not vary significantly in the different tissues studied.

The moisture content of the eight normal liver tissues varied from 54 to 69 per cent. The values for those livers removed from mice 3–16 weeks after repeated carbon tetrachloride feeding fell within this range. Four of five hepatomas for which moisture content was determined had higher values than the adjacent cirrhotic tissue.

Acid phosphatase activity was lower in the group of mice that had received 16 weeks' treatment on the basis of wet weight, dry weight, and lipid free dry weights as well as total tissue nitrogen (Table 1). Hepatomas showed no consistent difference. No significant consistent differences were noted in the content of succinoxidase in normal, cirrhotic, or hepatomatous tissue.

The data on lipids, alkaline phosphatase, and nucleic acids will be discussed with the respective

histochemical observations. The studies of nitrogen and phosphorus distribution showed a considerable range in the eight normal mice, from which the small number of samples of other tissues did not deviate significantly.

Alkaline phosphatase.—Gomori's method was used to demonstrate alkaline phosphatase histochemically in tissues fixed in absolute alcohol. In the control mice on fast, only the nuclei of the peripheral part of the lobule showed a weak alkaline phosphatase activity, while the central part gave a negligible response. An early change after carbon tetrachloride treatment was the intense reaction usually associated with inflammatory cells and a weaker reaction in the central degenerating area. Even later, after cirrhosis had

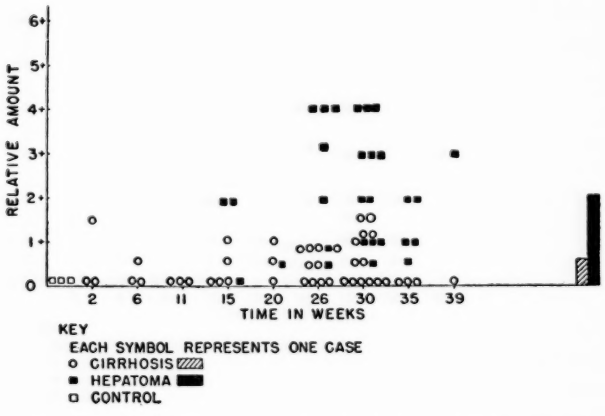

CHART 1.—Relative amounts of alkaline phosphatase visualized histochemically in cirrhotic and hepatoma tissues at intervals after repeated feedings of carbon tetrachloride. Mean values are shown on right of graph.

been induced, some areas of stronger reaction could still be seen, although generally the reaction was weaker in later stages (Fig. 5). In some cases no reaction could be visualized.

The alkaline phosphatase reaction in cirrhotic tissue was usually found in the nuclei and nucleoli. The cell membranes of compressed atrophic cells near hepatomas and those of necrotic cells might show a moderate reaction, but the cytoplasm in the cirrhotic part showed no activity. This was contrary to the changes observed in hepatomas.

In hepatomas the alkaline phosphatase activity also showed variation. Some hepatomas could be recognized easily by their intense reaction (Fig. 5). Some showed only a moderate reaction with the standard incubation period of 1.5 hours. However, the difference between hepatoma and cirrhotic tissue usually became more apparent if the incubation time was prolonged. In this series, over four-fifths of the hepatomas gave a stronger reaction than did the nearby cirrhotic tissue (Chart 1). Furthermore, the distribution of alkaline phosphatase was even more characteristic, for not only did the nuclei give a strong response, but also the cell membranes, nucleoli, and even the cytoplasm sometimes showed a strong reaction. In this experiment negligible alkaline phosphatase reaction was found in the cytoplasm, except in hepatoma cells.

Quite frequently, the cells near a strongly positive blood vessel showed a stronger reaction than did other parts (44). However, no difference was observed in the reaction of the blood vessels of tumors and of normal tissues as has been reported by Manheimer and Seligman (24). No consistent relationship between the intensity of the alkaline phosphatase reaction and the number of bile ducts could be found; as stated before, there were fewer bile ducts in hepatomas. After longer incubation in the substrate, many small, round structures, strongly suggestive of bile capillaries, could be seen between liver cells. This reaction is less intense in hepatomas than in the starved controls and in some cirrhotic tissue, and also much weaker than the reaction of nuclei and nucleoli in hepatomas.

No close relationship was noticed between the intensity of reaction and either the duration of carbon tetrachloride treatment or the size of the hepatoma. On the other hand, the size of hepatoma cells appeared to be an important factor. Most of the hepatomas consisting of large vacuolated cells showed a much stronger reaction than the nearby cirrhotic tissue. The hepatomas composed of smaller cells were less consistent, some showing stronger and some weaker reactions than the cirrhotic tissue.

The results of the chemical studies of alkaline phosphatase (Table 1) indicated an apparent increase after 16 weeks. The average value for hepatomas, as related to tissue nitrogen, was about a third more than in the adjacent cirrhotic tissue, although there was considerable individual variation among specimens. However, little difference was found chemically in the levels of this enzyme between hepatomas and normal liver.

Esterase.—Small, thin pieces of tissue were fixed in chilled acetone and stained by Gomori's method. Sections were usually incubated for 15 hours and counterstained with hematoxylin. Esterase activity was usually found in the cytoplasm but not in the nuclei. Calcified and necrotic areas gave a black lead precipitate in control sections; however, this reaction was different from the less intense black, fine granules of esterase. Variation of incubation time from 15 hours to 24 hours did not change the reaction noticeably.

Normally, only the cytoplasm of the liver cells of the central part of the lobules gave a strong reaction. During the first two weeks the esterase

activity showed a definite decrease (Chart 2), which is probably due to liver damage. However, in later stages when cirrhosis and hepatomas were found, the activity was extremely variable. The distribution within the lobule was no longer regular, and no definite relation to blood vessels or bile ducts could be noticed. This individual variation was striking and may explain the conflicting results of previous investigators.

The average esterase activity in cirrhotic tissue was similar to or a little above normal, while that of hepatoma cells was greatly reduced. This picture was not so consistent in individual cases. In some sections, both parts occasionally showed a similar degree of reaction, but in about three-fourths of the instances the reaction of the hepatoma was weaker than that of the cirrhotic tissue. This difference was marked in a few cases (Fig. 4). The size of the cells in hepatomas was not correlated with esterase activity.

The chemical studies of liver esterase (Table 1) confirmed the variation noted histochemically but did not show any consistent difference between hepatomas and the adjacent cirrhotic tissue.

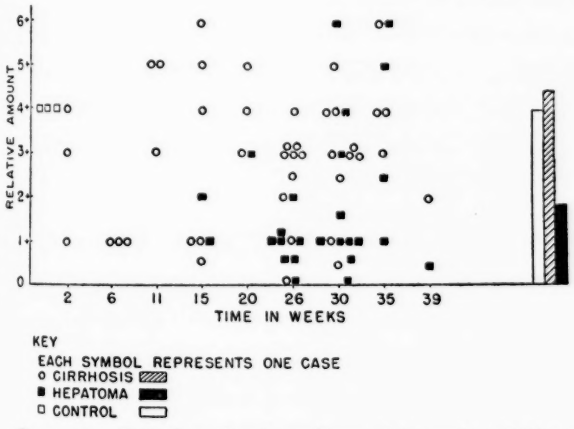

CHART 2.—Relative visual readings of intensity of histochemical reactions for lipase in tissues. Mean values are indicated at right margin.

Glycogen.—Tissue fixed in absolute alcohol was stained for glycogen by Gomori's method; the Hotchkiss technic was used occasionally and gave similar results. Control preparations were digested with saliva. The incubation time for Gomori's technic was 2 hours at 45° C. The livers of all control mice on fast contained only traces of glycogen around portal canals. In contrast to these controls, most of the experimental mice showed some glycogen in their liver cells. As can be seen in Chart 3, individual variation was conspicuous. However, there was a general tendency toward increased amounts of glycogen, which was more marked in the later stages of cirrhosis. Early cases usually showed weak reactions for glycogen.

Hepatomas showed more reaction for glycogen than cirrhotic tissues, and with few exceptions the latter had more than the controls. About nine-tenths of the tumors had a more intense glycogen stain than the adjacent cirrhotic areas. This observation suggests that individual nutritional factors are not the principal determinant for the amount of glycogen present in different areas. Although some cases occur in which the reactions of the hepatomas and the cirrhotic areas are equal, more often differences between areas of the same liver are marked. Furthermore, the transition from a strongly reacting hepatoma nodule to a weaker cirrhotic area was sometimes abrupt (Fig. 6).

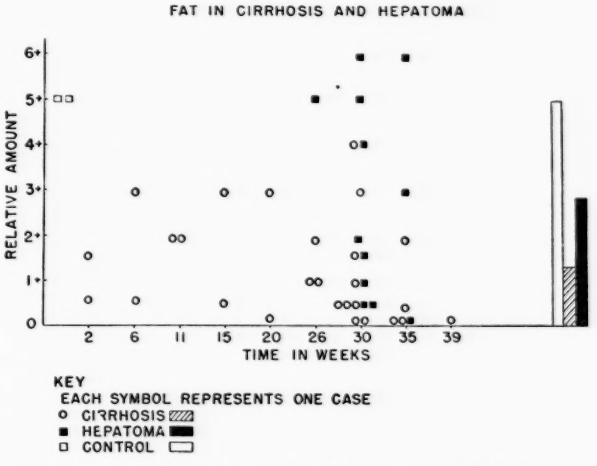

CHART 3.—Relative intensity of glycogen staining reactions in cirrhosis and hepatomas. Mean values given on right side.

There was no correlation between the glycogen and the size of the tumor, although there was less variation in the later stages. However, the size of the tumor cells was a definite factor. The large, vacuolated hepatoma cells almost invariably showed a moderately high glycogen content. The difference between such hepatomas and neighboring cirrhotic areas was apparent. On the other hand, the glycogen content in the small nonvacuolated hepatoma cells may occasionally be lower, although it was usually higher than that of neighboring cirrhotic tissue.

The distribution of glycogen in cirrhotic tissue showed a rather close spatial relationship to blood vessels. In some cases, only the liver cells that surround the portal canals showed a reaction for glycogen. This relationship was not so definite in hepatomas. Usually only the cytoplasm reacts in both cirrhotic tissues and hepatomas. Nuclei were only rarely positive. In a few hepatomas containing much glycogen, Kupffer's cells also showed some reaction.

The alkaline phosphatase and glycogen reactions of cirrhosis and hepatoma showed somewhat parallel changes. Both reactions were usually

strong in hepatomas, less in cirrhosis, and least in controls. In some cases, the localization of the two reactions was similar in adjacent sections from the same block, as shown in Figures 5 and 6. However, these results must be interpreted cautiously. Even though the glycogen content in cirrhotic tissues and hepatomas is higher than in the controls, it does not necessarily mean that their glycogen synthesis is increased. As all animals were on fast for 18 hours, the control value is the low residue after 18 hours of catabolism. It is possible that the higher value in the cirrhotic and tumor tissue represents a reduced ability to remove or utilize the glycogen, rather than an increased glycogen production. Because of the limited amounts of tissue in many of the samples, chemical measurements for glycogen were not made.

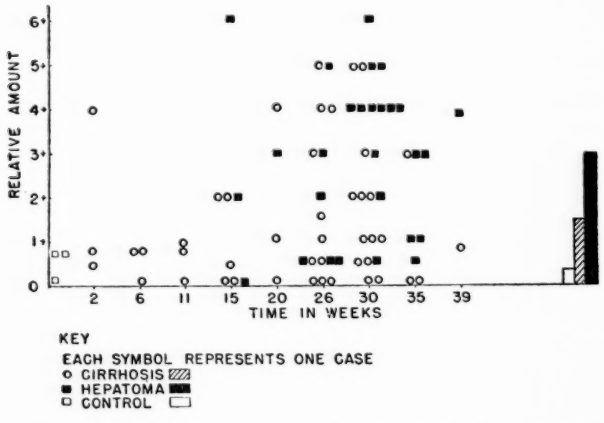

CHART 4.—Relative amounts of stainable lipids in cirrhosis and hepatomas following carbon tetrachloride feeding note. Mean values indicated at right margin.

Lipids.—Formalin-fixed sections were stained with oil red O and counterstained with hematoxylin and eosin. The results are summarized in Chart 4. The starved control mice showed the usual changes associated with starvation in this species. The fat content was invariably high, and the distribution diffuse.

In cirrhotic livers, there was less fat than in the control tissues. This extremely low fat content is peculiar to cirrhosis induced by carbon tetrachloride. In other forms of cirrhosis the fat content is increased. It has been assumed by some that severe fatty metamorphosis is the precursor of cirrhosis (3, 16). However, this fatty change apparently is not prominent in the cirrhosis produced by carbon tetrachloride.

On the other hand, hepatoma cells often contain increased amounts of visible fat. In this respect, the difference between hepatomas and adjacent cirrhotic tissue is sometimes striking. A clear demarcation between the two is often demonstrated. Some hepatomas contain little fat and are similar to cirrhotic livers. The average content of stainable lipids in hepatomas is still much below that of the fasting controls. The fat content cannot be correlated with the size of the hepatomas, but, as in the case of glycogen and alkaline phosphatase, the size of the cells in the hepatoma is an important factor. Most of the large, vacuolated hepatoma cells appear to contain less fat, whereas the majority of small cells contain larger amounts of fat. This relationship is the reverse of that shown in glycogen studies in which there is a higher glycogen content in the larger cells. Frequently, the distribution of fat in cirrhotic tissue is perivascular, so that when there is little fat it is found only in the cells around the blood vessels. This unusual pattern is not usually seen in hepatomas, where the fat distribution is either diffuse or patchy and without apparent vascular relationship.

In many hepatomas, and sometimes in cirrhotic livers, a few nuclei show distinct red-stained fat droplets, which rarely occupy most of the nucleus[1] (38). The fat is not consistently associated with nucleoli or with the so-called nuclear inclusion bodies which are free from fat in this series. The cells whose nuclei contain fat usually have their cytoplasm loaded with lipids. This nuclear change is found often in the large vacuolated cells. Morphologically, the nuclei containing fat may be normal, but in many cases these nuclei are larger and more granular than those in other cells.

As determined chemically, the lipids of the liver after 3 weeks' treatment with carbon tetrachloride decreased 59 per cent from normal on the basis of wet weight (Table 1) and 49 per cent on the basis of dry liver weight. The lipid content of the tissue remained low throughout the development of cirrhosis. The average cholesterol content of the liver was 6.52 mg/gm wet liver in controls, which value decreased to an average of 4.8 by 12 weeks and increased to 7.88 at 16 weeks. Insufficient tissue was available for lipid analyses on most of the hepatomas.

Nucleic acid.—The Feulgen reaction on tissue was used to demonstrate desoxypentosenucleic acid (35); pyronin-methyl green (32, 40) and ribonuclease (39) were used for pentosenucleic acid studies.

The ribonucleic acid of control liver tissue from animals on fast and cirrhotic tissue usually showed a staining gradient within the lobule, being more intense in the periportal area and less so in the center. In hepatomas this lobular pattern was no longer recognizable. The reaction may be either diffuse throughout the tumor, or it may

[1] B. Brock, R. E. Stowell, and K. K. Couch, observations to be published.

vary from one area to another without demonstrable relationship to blood vessels. Most of the thread-like pyroninophilic structures found around the normal nucleus were decreased or converted into a rather homogeneous material. The amount of ribonucleic acid and the size of hepatoma cells show some correlation. The small hepatoma cells frequently give a stronger reaction while the large vacuolated cells more often have a less intense reaction, in comparison with the cells of the neighboring cirrhotic tissue. The weaker reaction of these large hepatoma cells may be due to a dilution effect by other cellular constituents.

The nuclei of cirrhotic tissue present a complex picture. In the early stages of cirrhosis there may be groups of large, irregularly shaped polyploid nuclei showing a strong Feulgen reaction, while other groups of nuclei in smaller cells show a weaker reaction. In later stages this picture becomes more simplified and uniform; by visual inspection the Feulgen reaction per area is relatively stronger in cirrhosis than in the controls.

In most of the hepatomas the intensity of the Feulgen reaction per field is also increased, although some of the enlarged nuclei appear to give a less dense reaction. It is possible, however, that this appearance is due to dilution; a weakly stained large nucleus may contain as much nucleic acid as a strongly reacting but smaller one.

Chemical measurements of desoxypentosenucleic acid (DNA) in Table 1 show an increase in the 12- and 16-week stages. There was considerable individual variation in the hepatomas and adjacent cirrhotic tissue, which was partially explained by the observed variations in cytoplasmic volume and numbers of fibroblasts and inflammatory cells.

The chemical determinations of pentosenucleic acid (PNA) in Table 1 showed no significant change during the development of cirrhosis. The values per unit tissue nitrogen for each of the eight hepatoma samples were higher than for the adjacent cirrhotic tissue. Chemical determinations for total nucleic acids, as measured by the specific carbohydrate, ultraviolet absorption, and phosphorus (41), showed good agreement.

DISCUSSION

Alkaline phosphatase.—The present experiments show clearly that alkaline phosphatase increases in both cirrhotic tissue and hepatomas. Recently, Sherlock and Walshe (33) reported increased enzyme in active cirrhosis but found no increase in what they call inactive cirrhosis. These observations were based on human material but are similar to the present data on animals.

The literature with respect to the alkaline phosphatase content of hepatomas is confusing. Different types of hepatomas, as well as different generations of serial transplants of the same hepatoma, give different chemical results (14). Transplants of spontaneous hepatomas in two strains of mice and of hepatomas induced by carbon tetrachloride and aminoazotoluene did not contain more alkaline phosphatase when determined chemically by Greenstein (14), whereas a hepatoma induced by chloroform did. The histochemical and chemical observations on this present series are not in complete agreement. Histochemically, most of the hepatomas showed more reaction than the adjacent cirrhotic tissue, and the normal liver showed a low response. Chemically, the hepatomas had a higher average alkaline phosphatase content than the cirrhotic tissue, but an amount equivalent to the normal average. Considerable individual variation was noted among different specimens by both methods of observation. The explanation of the differences between the chemical and histochemical observations on normal liver is not evident at this time.

The increased activity in the hepatomas of mice in this series is in agreement with the findings in hepatomas of rats (12), although the latter usually give a much more intense reaction. It has been suggested that this species difference in phosphatase activity may be related to the presence of the gall bladder in the mouse and its absence in the rat.

Some investigators believe that increased alkaline phosphatase in the liver is due to bile obstruction. Therefore, it was desirable to study the bile capillaries in hepatomas, since the increased enzyme activity might be due simply to retention of bile. However, in the present series there was no microscopic evidence of marked bile retention or bile pigment in hepatoma nodules. Furthermore, in a series of sections showing a strong alkaline phosphatase reaction, cross sections of bile capillaries showed a moderate reaction, which suggests that they were functioning; however, this reaction was much less intense than that of the nuclei and nucleoli. These observations make it difficult to explain the high phosphatase activity of hepatomas on the basis of bile obstruction.

Liver esterase.—Relatively few studies have been made on the function and changes of liver esterase during liver diseases. Whipple (46) and others have shown that the blood esterase increases greatly when liver cells are damaged. Greenstein found that esterase decreased in various tumor tissues, although the reduction of esterase in hepatomas was not so marked as in other tumors. Jobling, Eggstein, and Petersen (18)

found a decreased level of liver esterase in association with liver damage.

Histochemical studies of the present series showed a reduction of esterase in hepatomas, in agreement with Greenstein's chemical results (13). Moreover, in some cases of cirrhosis there is an almost equal reduction of activity, although the picture is less uniform than in hepatomas. In the few chemical measurements of esterase a different substrate was used than that employed for histochemistry, and it showed more evidence of variation in different tissues than of consistent changes. The large individual variation in results of chemical studies (Table 1) in different mice of presumed comparable condition is of considerable interest.

Glycogen.—Histochemical estimations of glycogen are not as quantitatively accurate as chemical analyses (11, 15), but they give better information on glycogen localization. Although extensive chemical analyses of carbohydrate metabolism of tumor tissue have been reported, few histochemical studies of glycogen have been made. It is necessary to use caution in evaluating quantitative glycogen changes demonstrated histologically. The frequent occurrence of high values in hepatomas, especially in the large cell type, is in sharp contrast to the absence of glycogen in control tissues. Orr and Stickland (30) present evidence that, although normal liver cells can break down glycogen for their energy supply, hepatoma cells derive energy mainly from glucose instead of glycogen. If this is true, more glycogen should be spared in hepatomas than in other parts of the liver, since the hepatoma cells could obtain glucose from the circulating blood. Furthermore, there may be an increase of glycogenesis in these tumor cells. Although this is possible, and an increase of glycogen production has been reported in other tumors (34), glycogenesis was not studied in these experiments.

Regardless of the explanation of the increase in glycogen, its concomitant occurrence with alkaline phosphatase activity is an interesting finding. Their co-existence has been reported in embryonal bone before ossification by Gomori (9) and others. Furthermore, some evidence suggests that alkaline phosphatase plays a role in carbohydrate metabolism. It has frequently been pointed out that the two organs primarily concerned with carbohydrate absorption and excretion, the intestine and kidneys, contain considerable amounts of alkaline phosphatase. It is thought that this phosphomonoesterase acts in the glycogen-glucose cycle. Therefore, the co-existence of alkaline phosphatase and glycogen in hepatomas may be significant.

It is well known that a high carbohydrate diet can protect liver cells against a variety of substances that produce liver injury, such as chloroform and infection (4, 29), yet there is little experimental evidence that the hepatic glycogen has an equivalent role (2). The comparatively higher glycogen content in hepatomas and in the later stages of cirrhosis might be associated with the resistance developed in some liver cells to carbon tetrachloride. The tissue apparently develops resistance to carbon tetrachloride after repeated treatments, and the glycogen content is considerably increased at the same time. Hepatoma cells show little response to carbon tetrachloride; for example, fibrosis is rarely seen in tumor nodules. In this case too there is a high glycogen content, which might be acting as a protective agent.

Lipids.—Since it is known that the nutritional condition of an animal influences the lipid stores of the liver, in order to study a more uniform metabolic state all the animals of the present series were put on fast before being killed. It was found that the cirrhotic and hepatoma tissues contain much less fat than the controls. Kretchmer and Barnum (20) reported a similar finding based on chemical determinations. They found that cirrhotic animals on fast show 1–2 times as much liver lipid as fed controls, while normal mice on fast for 24 hours have 3–4 times more liver lipids.

Ceroid pigment.—The characteristics of ceroid in the present series have been discussed elsewhere (21). This pigment is often seen in cirrhotic tissue but not frequently in hepatomas. The evidence suggests that ceroid represents a product of cellular destruction.

Characteristics of cirrhosis and hepatomas induced by carbon tetrachloride.—The nonportal fibrosis found in experimental cirrhosis has been well demonstrated by Ashburn, Endicott, Daft, and Lillie (1). In the present series the fibrosis can can also be traced to the central vein rather than to the portal veins in the early stages. However, later, when fibrosis is widespread and uniform, the portal as well as the central vein is involved.

There is evidence of some degenerative processes in the early stages of cirrhosis, but none are found in later stages. This again suggests that some kind of cellular resistance develops during the course of treatment. Some of the long-term cirrhotic tissues may even present a glycogen and fat picture similar to that of normal control liver.

Because of the recognized hepatotoxic, necrosis-producing action of carbon tetrachloride, the induced cirrhosis is usually referred to as a form of

post-necrotic cirrhosis similar to that seen in subacute yellow atrophy. However, the following observations raise some questions relative to this interpretation. There is not much necrosis in the later stages of cirrhosis, even when carbon tetrachoride is given continuously. Also, there are no areas of perfectly normal-looking liver cells, as is the case in typical post-necrotic cirrhosis. An example of the latter is found in the cystine-deficient rat (17). Finally, the newly formed pseudo-lobules in the present series are fairly uniform in size, which is characteristic of monolobular cirrhosis; multilobular cirrhosis is more characteristic of post-necrotic cirrhosis, such as subacute yellow atrophy.

It is not necessary to produce cirrhosis for experimental hepatoma formation. These two diseases have been obtained separately (28). However, cirrhosis and hepatomas frequently occur together. This coincidence may mean that both conditions are due to a common cause or that they represent stages of the same pathological process. The histochemical observations of the present series give information related to this problem. The fat changes were irregular and did not follow any clear pattern. However, the study of other chemical constituents reveals that normal liver and hepatomas usually represent two extremes as far as the amount or activity of the constituents is concerned. Furthermore, the values in cirrhotic liver are frequently intermediate between those of normal and hepatoma tissues. At present, the relationship between these stages is not well enough understood for one to draw definite conclusions.

When carbon tetrachloride feeding causes necrosis in the liver, the liver shows tremendous regenerative powers. Therefore, the possibility that tumors produced by carbon tetrachloride are regenerative nodules must be considered. The work of Eschenbrenner and Miller (6) clarifies this problem considerably. First, they showed that a long-term treatment with small non-necrotizing doses of carbon tetrachloride which do not produce visible damage in the liver can produce hepatomas. Secondly, they demonstrated that after several feedings, even with the usual dose, there is no visible damage after further injections. Therefore, necrosis is apparently not necessary for hepatoma formation.

There is no doubt that the carbon tetrachloride treatment is related to the production of hepatomas. The incidence of spontaneous hepatomas in strain A mice is negligible. Furthermore, forced feeding of olive oil alone never produces tumors.

There is considerable evidence supporting the neoplastic nature of these hepatomas, which generally are benign rather than malignant. The finding of acinous structures not resembling bile ducts in several cases, and an angiomatous growth in one case, is suggestive of neoplasia. Previous investigators (5) have proved that carbon tetrachloride hepatomas can be transplanted, which again is a criterion of neoplasia.

SUMMARY AND CONCLUSIONS

Some of the changes in mouse livers after repeated bi-weekly feedings of carbon tetrachloride were studied by histochemical and microchemical methods during the development of cirrhosis and in hepatomas. Samples of liver tissue from various mice, and hepatomas from the same and different mice, showed considerable variation.

The liver cells that survive a single injection of carbon tetrachloride show evidence of a disturbed metabolism of glycogen and lipids. Glycogen is increased in the later stages of cirrhosis and in most hepatomas. The lipid content of both cirrhotic and hepatoma tissues is much less than that of normal liver from animals on fast. Some of the hepatomas had more lipids than the adjacent cirrhotic tissue. Fat was observed in the nuclei of some liver cells. The cholesterol content of the liver decreased during the third- to twelfth-week stages of cirrhosis development. Alkaline phosphatase is usually increased in cirrhotic tissue and to an even greater extent in hepatomas. The water content of the hepatomas was higher than in the cirrhotic liver.

In cirrhotic and hepatoma tissue the content of both the nucleic acids varies in individual cases. The observed increase in desoxypentosenucleic acid in some cirrhotic livers and hepatomas may be explained by the increased content of nuclei of fibroblasts and inflammatory cells. Chemical measurements of pentosenucleic acid were higher in hepatomas than in adjacent cirrhotic tissue.

Histochemical observations showed esterase to be slightly increased in cirrhosis and frequently decreased in hepatomas, whereas the chemical findings were variable in all tissues. The chemical studies of nitrogen, phosphorus, acid phosphatase, and succinoxidase did not show significant changes.

A yellow pigment similar to ceroid was observed in both the short- and long-term studies. Occasional heterotopic bone formation in cirrhotic areas was noted.

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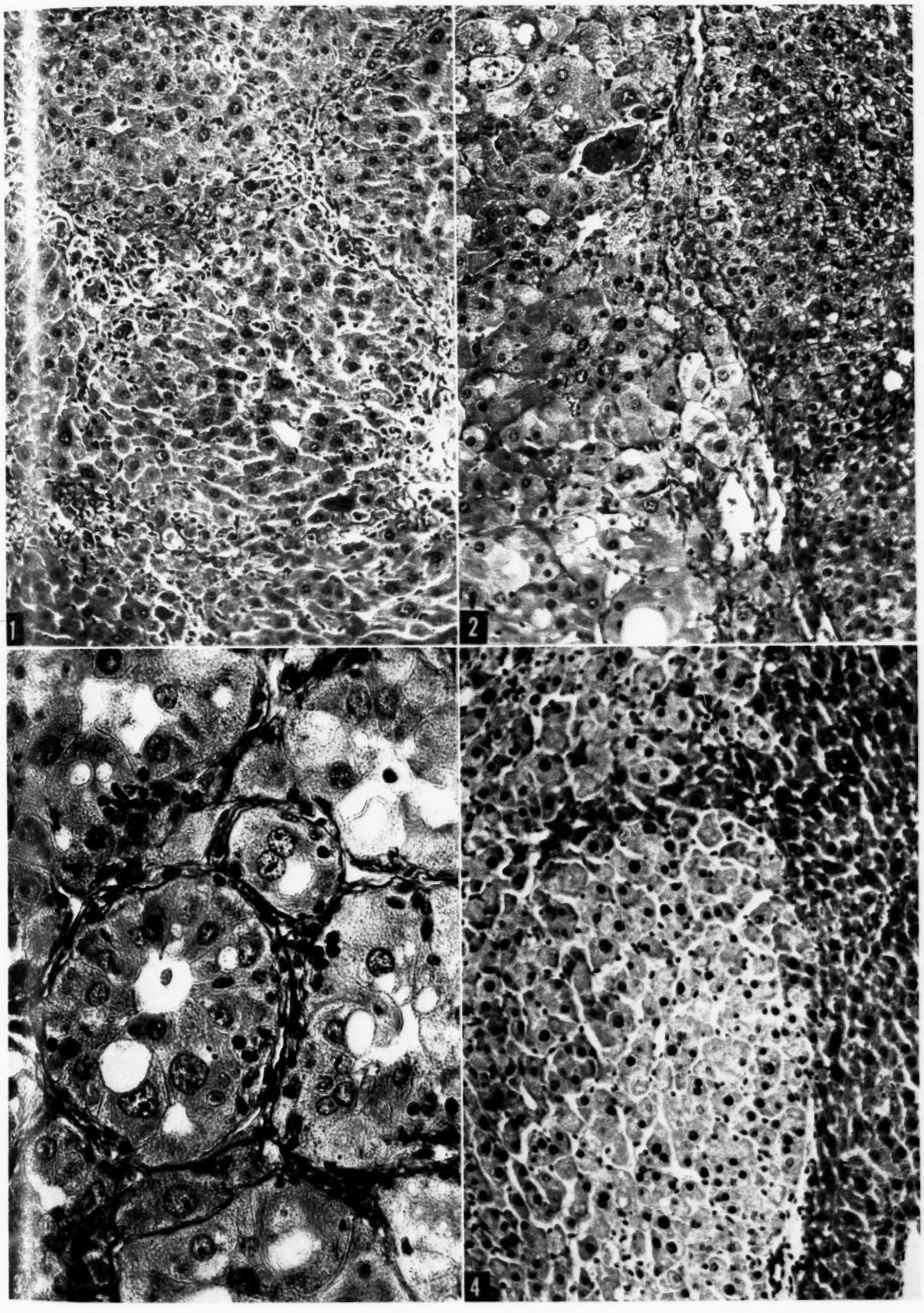
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FIG. 1.—Fibrosis of mouse liver induced by repeated feedings of carbon tetrachloride. Heidenhain's azocarmine stain. Mag. ×175.

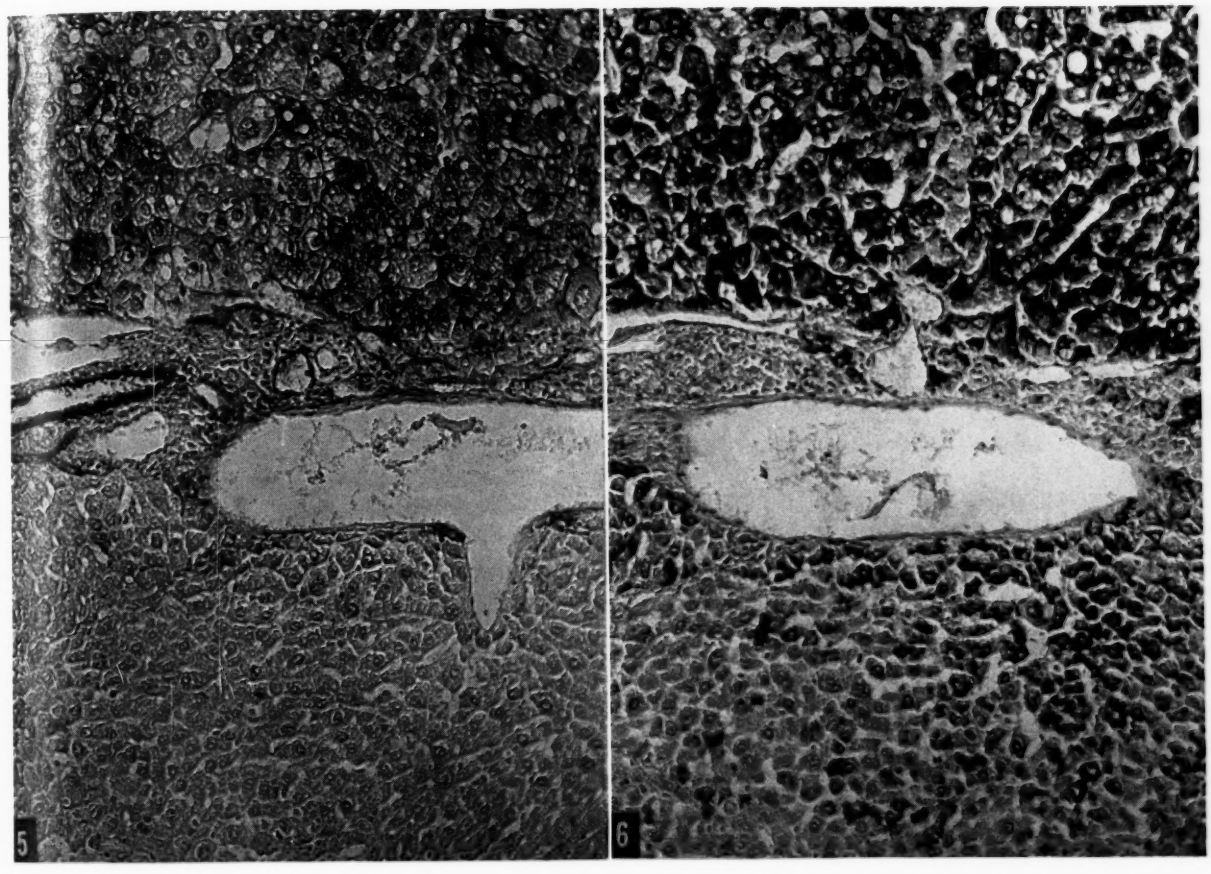
FIG. 2.—Junction between hepatoma (on left) and mouse liver. Hepatoma shows loss of lobular pattern and cells have more abundant cytoplasm. Heidenhain's azocarmine stain. Mag. ×175.

FIG. 3.—Acinous structures present in part of hepatoma shown in Figure 2. Heidenhain's azocarmine stain. Mag. ×475.

FIG. 4.—Hepatoma (on left) and adjacent liver tissue stained with Gomori's reaction for lipase and nuclei counterstained with hematoxylin. Mag. ×175.

FIG. 5.—Alkaline phosphatase reaction on hepatoma (above) and adjacent liver. Mag. ×175.

FIG. 6.—Hepatoma (above) and adjacent liver stained for glycogen by Gomori's method. This section was near that shown in Figure 8. Mag. ×175.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Oxidative Phosphorylation in Homogenates of Normal and Tumor Tissues*

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Earlier studies from this laboratory (13, 18, 20) with homogenates prepared in isotonic KCl showed that tumor tissues were essentially devoid of oxidative activity in reaction mixtures in which large quantities of oxygen could be used by homogenates of brain, heart, liver, and kidney. The circumstances of the oxidation were such that it might be assumed that the oxalacetate, when oxidized, proceeded via the Krebs citric acid cycle and that the process was phosphorylative in nature. Studies with kidney (13, 18, 20) showed that the oxidations are maintained by the phosphate reservoirs which they in turn maintain, and it therefore became desirable to correlate the oxidative rates in the various tissues with the status of the phosphate reservoir during the course of the reaction, since failure to maintain the reservoir might result in a loss of oxidative activity before measurement of it could be made. In addition, the nature of the oxidative pathway was studied by determining the levels of intermediates in the Krebs citric acid cycle. Malic and citric acids were determined, since the metabolic pools of these substances in an equilibrium system would be much greater than the amounts of the other intermediates. Oxalacetate was the substrate added, and the rate of its disappearance was followed analytically.

EXPERIMENTAL

Materials and Methods

Preparation of tissues.—The tissues were obtained from young, adult male rats weighing between 200 and 300 gm. The animals were obtained from the Holtzman Rat Company and were maintained on a stock diet of mixed grains for 1–2 weeks before they were used. The rats were killed by decapitation, and the tissues were chilled in ice-cold isotonic KCl. The tissues were blotted, weighed, and homogenized in cold isotonic KCl. All-glass homogenizers were used to avoid metal contamination and to maintain nuclei and mitochondria as intact as possible.

Measurement of oxalacetic oxidase activity.—The enzyme activity measurements were carried out under the conditions described by Potter *et al.* (20) with occasional slight deviations. In all cases 1.5 ml. of a stock solution similar to that described earlier (20) was added to each flask to give a final concentration of 0.067 M KCl, 3.3×10^{-3} M $MgCl_2$, 6.6×10^{-3} M potassium phosphate, and 1.3×10^{-5} M cytochrome c at pH 7.0. Other properties of the system and precautions as to speed and cold, proper tonicity, use of alkaline KCl, and partially neutralized oxalacetic acid (13.3 μM per flask) have been noted in earlier publications (18, 20). The ATP was added to give a final molarity of 0.001 M in a volume of 3.0 ml. The oxygen uptake was measured at 10- or 20-minute intervals in duplicate flasks, and all the data are recorded in terms of the oxygen uptake per 10 minutes, plotted against time, in order to correlate the decline in oxidative enzyme activity with changes in the concentrations of primary substrate, intermediates in the Krebs cycle, inorganic phosphate, and ammonia.

Analytical methods.—Protein-free filtrates were prepared by adding 2.0 ml. of cold 17.5 per cent trichloroacetic acid per Warburg flask to give a final volume of 5.0 ml. and a trichloroacetic acid concentration of 7 per cent. The flasks were removed from the manometers at various intervals and placed in an ice bath immediately before adding the acid (14). Analytical methods were as follows: inorganic phosphate (8), citric acid (9, cf. 20), malic acid,[1] ketoacids (5, cf. 20), and ammonia (1).[2]

Reagents.—Oxalacetic acid was prepared by Dr. C. Heidelberger, using an improved method

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[1] J. F. Speck, unpublished data.

[2] We are greatly indebted to Drs. John W. Harmon and Jean H. Webster of the Pathology Department for making preliminary determinations and for advice on the use of this procedure.

of synthesis (6). Pyruvic acid was redistilled *in vacuo*, maintained as a 1 N solution in the cold, and neutralized with K_2CO_3 before use. ATP was prepared from rabbit muscle under the direction of Dr. G. A. LePage (cf. 24). Cytochrome was prepared from beef heart according to the method of Keilin and Hartree (cf. 24).

RESULTS

A series of determinations of oxygen uptake was carried out with 12–16 identical reaction mixtures containing aliquots of the same tissue homogenate. After 10, 30, 50, 70, 90, and 120 minutes, successive pairs of duplicate flasks were treated with trichloroacetic acid and analyzed for ketoacids, citric acid, malic acid, inorganic phosphate, and ammonia. In subsequent experiments which were extended to as much as 300 minutes, the flasks were taken off at somewhat different intervals. The experiments of long duration were continued in order to reach the plateau level of phosphate and ammonia output. Each tissue was tested several times in experiments in which all the analyses were performed on the same reaction mixtures. Numerous experiments in which only part of the analyses were done are unreported, but they support the data in Charts 1–5, in which the results of 2–7 experiments on each tissue have been averaged. The data have been recorded graphically to show the time course of the changes in the reaction mixture and to facilitate comparisons between tissues. Instead of plotting all the changes for each tissue in one chart, the data on each reaction component that has been determined are plotted in separate graphs that show the changes when the various tissues were used. The results for each of the reaction components correlate very satisfactorily with changes in the other reaction components for each of the tissues studied, and may be considered in terms of their interrelationships. Thus, the oxygen uptake data (Chart 1) are related to the data on substrate disappearance (Chart 2) and on intermediary metabolite formation (Chart 3), while the inorganic phosphate changes (Chart 4) are related to both oxygen uptake and metabolite data, and the ammonia data (Chart 5) are secondary to the phosphate data. A typical experiment on kidney is shown in Chart 6, in which all the measurements have been recorded in a single chart to show the correlation among the various findings.

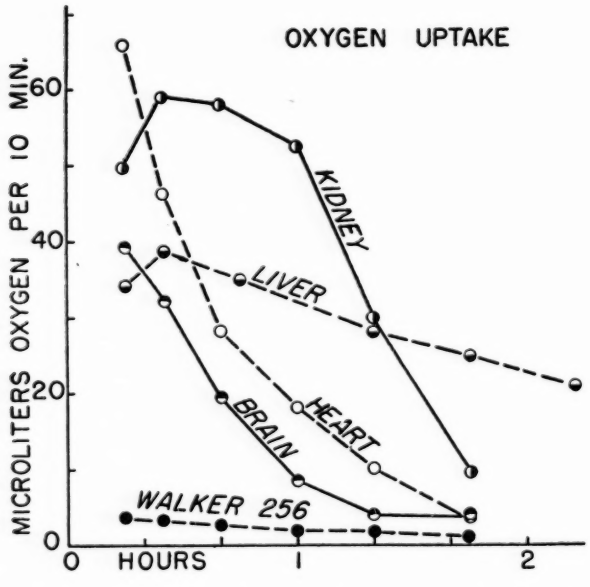

CHART 1.—Changes in rate of oxygen uptake by whole homogenates of various rat tissues with oxalacetate as the added substrate.

Conditions as described in text. The amount of tissue per flask was as follows: kidney, 30 mg.; heart, 40 mg.; and liver, brain, and tumor, 50 mg. Each curve is the average of two to four experiments with homogenates from different rats. The following four figures represent the results of analyses from experiments that included those reported in this figure.

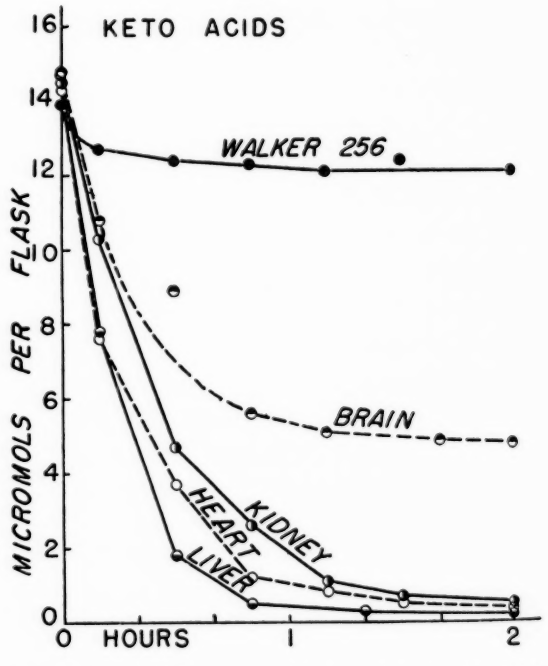

CHART 2.—Disappearance of ketoacid during the oxidation of oxalacetate as shown in Chart 1.

The data on oxygen uptake are given in Chart 1 and may be compared to the data on ketoacid disappearance in Chart 2. Since in these experiments oxalacetate was the only substrate added, and since the conversion of oxalacetate to pyruvate does not involve ketoacid disappearance, Chart 2 is a record of substrate utilization. The accumulation of α-ketoglutarate is negligible under these conditions. It may be seen that oxalacetate was rapidly utilized by homogenates of heart, liver,

kidney, and brain but that in the latter the oxygen uptake ceased before the ketoacid was used up after a period of rapidly declining oxygen uptake. In the case of the Walker 256 carcinosarcoma, no period of oxygen uptake was observed, and there was a very slight disappearance of ketoacid in the first 20 minutes of incubation. Identical results

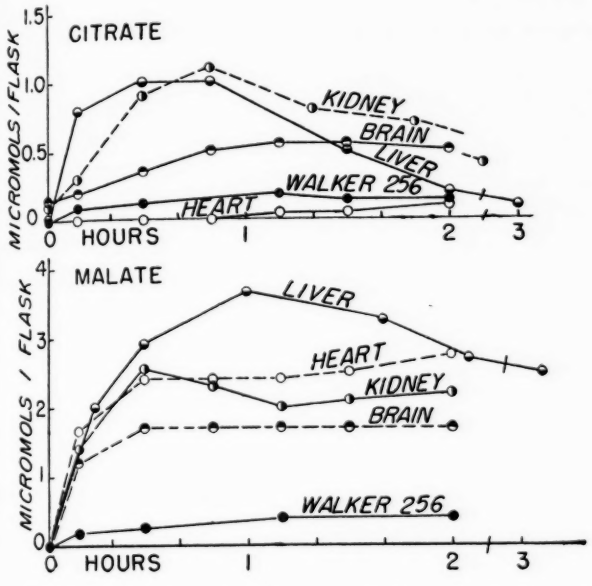

CHART 3.—Changes in the concentration of citrate and malate during the oxidation of oxalacetate as shown in Chart 1.

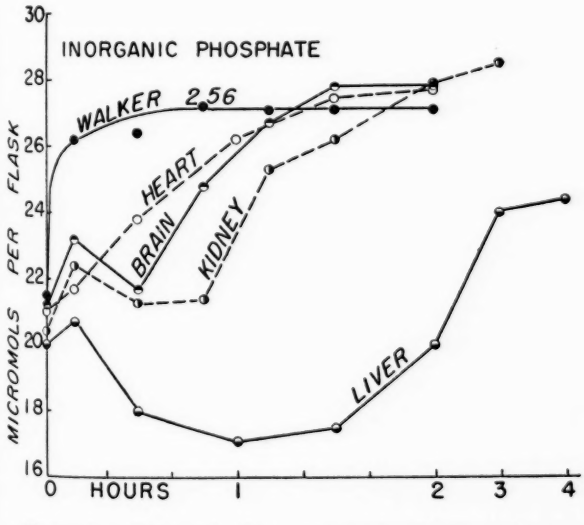

CHART 4.—Changes in the concentration of inorganic phosphate during the oxidation of oxalacetate as shown in Chart 1.

were obtained with Flexner-Jobling rat carcinomas. It appears that in brain there was an active enzyme system initially, but the system became inactivated rapidly and was no longer capable of utilizing ketoacid, while in the tumor tissue the amount of enzyme was much smaller initially, or it was inactivated even sooner. In both tissues, there was essentially no change in the level of ketoacid during the second hour of incubation. In heart homogenates the decline in oxygen uptake paralleled the decline in ketoacid, but in liver and kidney homogenates there was a considerable

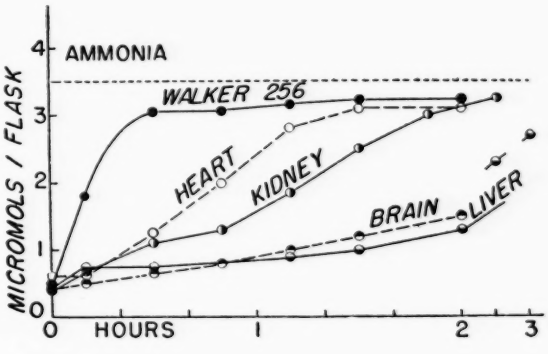

CHART 5.—Changes in the concentration of ammonia during the oxidation of oxalacetate as shown in Chart 1.

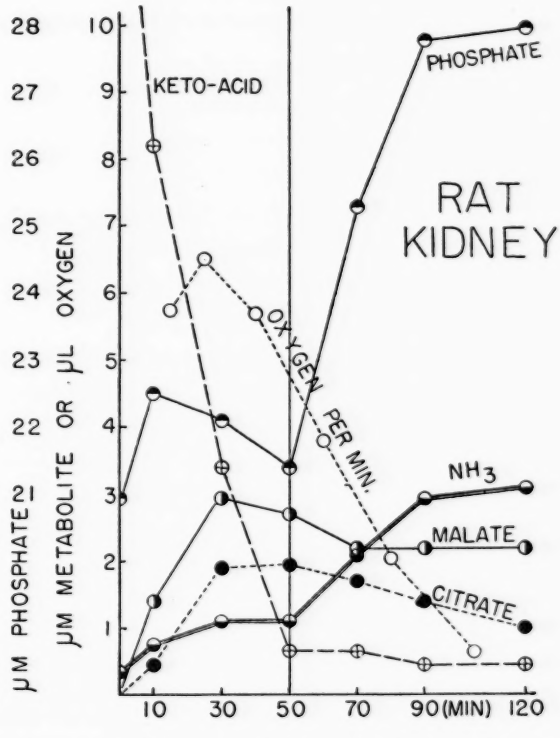

CHART 6.—Typical experiment with 30 mg. rat kidney, showing correlations between chemical changes occurring in the reaction mixture. Phase I is shown during 0–10 minutes, phase II, 10–50 minutes, and phase III, 50–120 minutes. Original oxalacetate concentration was 13.3 μM.

amount of oxygen taken up after the ketoacid level had fallen to less than 1 μM per flask.

A partial explanation for the continuation of oxygen uptake in the liver and kidney homogenates may be seen in Chart 3, which shows the changing levels of citrate and malate during the incubation period. There was an increase in these

two metabolites during the period when ketoacid disappearance was very rapid, and after the ketoacid level reached values of 1–2 μM per flask (or about 0.0005 M), the citrate and malate levels no longer increased, but declined somewhat in conjunction with continuing utilization of oxygen—notably in liver. In heart and brain there was no decline in malate or citrate after the ketoacid utilization ceased, and thus after the first hour the homogenates of these tissues were essentially inert as far as fuel combustion was concerned.

The ability to reduce ketoacid concentration to very low levels and to continue to oxidize the citrate and malate is definitely correlated with the maintenance of a favorable phosphate balance as shown in Chart 4. The homogenate of tumor tissue did not oxidize ketoacid and therefore did not maintain ATP; a rapid output of inorganic phosphate was observed. In brain, kidney, and liver homogenates there was an initial output of phosphate during the period of malate accumulation, when the oxalacetate was at its highest concentration and was competing with oxygen for the available hydrogen and electrons. This was followed by an uptake of phosphate, as the oxidative phase became dominant, and finally by an output of phosphate as the ketoacid was used up. These three phases were noted earlier in *kidney* homogenates by Potter *et al.* (18), but at that time the data on the oxidizable substrates and ammonia were not available. The reversals in the trend of the phosphate curve, although seen as single points in Chart 4, are not experimental errors, since experiments with closely spaced points have shown that true reversals occur as indicated (cf. Chart 5 and [18]). The failure of the oxidative mechanism is nearly simultaneous with the change in the phosphate balance from uptake to output, and comparison of Charts 2–4 shows that after the phosphate output of phase III sets in, the metabolites are no longer oxidized. The case of liver is of special interest, because the period of phosphate uptake (phase II) is prolonged and because the phosphate level does not plateau during phase III at the level that it does with the other tissues. It is also seen that with liver there is an absolute fall in inorganic phosphate that approximates the difference between the final values obtained for liver and the other tissues. It seems possible that the data may reflect the formation of inorganic pyrophosphate during phase II in liver in view of previous reports (cf. 2, 3). The source of the phosphate in these mixtures is ATP plus a small amount of organic phosphate present in the homogenate. Measurements of total phosphorus (18) showed that in kidney the phosphorus released from the homogenate is quantitatively small in comparison with the total in these experiments. The curves for ammonia formation (Chart 5) are closely correlated with the phosphate curves of Chart 4, with the exception of the brain homogenates. In the other tissues, ammonia is given off shortly after the phosphate is split off, but in brain the ammonia output is very slow. In kidney, the ammonia output occurs only during phases I and III, and the concentration of free ammonia remains constant during phase II when inorganic phosphate is being removed from the reaction. This stationary phase of the ammonia curves in kidney homogenates is much more pronounced in individual experiments, e.g., as shown in Chart 6, than in Chart 5, which represents the average of five homogenates. The data suggest that ATP, as such, cannot be deaminated, and that deamination occurs only after at least one phosphate has been removed from the ATP molecule. The removal of ammonia is evidently not part of the dephosphorylation reaction, because in brain the phosphate output is not accompanied by a corresponding output of ammonia. Furthermore, the two reactions can be dissociated by fractionating the homogenate, as shown elsewhere (19).

DISCUSSION

The data in the present study show that a valid assay for the oxidizing enzymes of the Krebs cycle can be obtained only during the period in which the phosphate balance is being maintained, and that in homogenates in which no phase II occurs the period of valid oxygen uptake rate may be very brief as in brain, heart, and tumor homogenates (Chart 1). In this chart, the data suggest that tumor tissue must have a relatively low level of the oxidative system, since it appears inactive at the outset. However, the data on ketoacid removal (Chart 2) show that there was a small initial period of activity in tumor homogenates during the interval before maximum phosphate output had occurred and before oxygen uptake measurement was begun. This *transient* period of ketoacid removal is in contrast to the slow but constant rate of extra ketoacid disappearance in glycolyzing tumor homogenates in which ATP was maintained (17). In the latter, the rate was comparable to that which had been observed in tumor slices (4).

The net conclusion from the data in Charts 1–5 is that in the tumor homogenates studied the breakdown of ATP is so rapid in these reaction mixtures that no oxidative activity can be expected. The next question is whether the reaction components might be altered so as to make conditions more favorable. In these experiments, oxalacetate was used as the sole substrate, be-

cause it could serve as a source of both oxalacetate and pyruvate, and its disappearance could readily be followed analytically. However, this compound is rapidly reduced to malate (Chart 3) during phase I, at which time the oxygen uptake in liver and kidney is submaximal and ATP breakdown occurs. This substrate thus appears to compete with oxygen, thereby lowering the yield of phosphate bond energy (cf. 7).

In earlier studies with water homogenates, in which the phosphatase action is more pronounced than in KCl homogenates (18), Potter (14) was able to convert a negative phosphate balance to a positive phosphate balance with sodium fluoride, which has frequently been used to cut down phosphatase activity in systems involving oxidative phosphorylation. However, in those experiments and in subsequent tests upon the improved kidney system (13), fluoride *decreased* the rate of oxygen uptake, and no attempt was made to increase the activity of inactive tumor homogenates by fluoride at that time. Meanwhile, other investigators, using isotopically labeled substrates with tumor slices, had shown that hepatomas could form CO_2 from glucose more rapidly than could normal liver (11, 27), while pyruvate was handled equally well by both tissues (11). Although the glucose finding may be related to a special inability of liver slices to burn glucose (10), recent studies with labeled fatty acids, including acetate (25), have shown that labeled citrate and CO_2 could be formed from these substrates in several kinds of tumor slices which were compared to liver and kidney slices. The occurrence of the "condensing enzyme," aconitase, and isocitric dehydrogenase was also demonstrated in these these (26), and earlier studies from the McArdle Laboratory had demonstrated significant activity for succinic and malic dehydrogenases and for hydrogen transport components. However, when slice studies with labeled acetate were begun here they showed an almost complete lack of the ability of two types of rat tumor to convert acetate to CO_2, as compared to liver and kidney (12). Studies with labeled pyruvates (carbonyl and carboxyl) were then undertaken, and it was found that in tumor slices pyruvate could be oxidized to CO_2 at rates that approached the magnitude seen with liver and kidney.[3] However, in contrast to the tumors studied elsewhere (25), only the hepatoma slices were active with acetate, and the previous observations with acetate (12) were confirmed. The pyruvate oxidation was carried out under a variety of conditions with *trans*-aconitate and malonate as inhibitors, and the results support the experiments of Weinhouse *et al.* (25, 26), in so far as they indicate the existence of a Krebs cycle in tumor tissues. These observations are in harmony with the findings in the present report in that both indicate that the homogenization process and subsequent conditions used previously have led to the loss of Krebs cycle activity from tumor homogenates but not from liver and kidney homogenates; but an important point to be settled is whether or not the slice technic can be taken to indicate the relative levels of the oxidative enzyme components in normal and tumor tissues, as it would seem likely that the ease with which the Krebs cycle activity is lost from the homogenate may be an indication of a low level of these enzymes as well as a high level of destructive enzymes. Separate studies with liver and kidney mitochondria and nuclei (19, 22) show that the level of oxidative activity is affected by the balance between phosphate breakdown and phosphate uptake, so that an inhibitor which affects this balance may stimulate in one system and inhibit in another (cf. 23). Thus, fluoride, which *inhibited* the kidney homogenate (see phosphate uptake in Chart 4), might *stimulate* the tumor homogenate (see phosphate output in Chart 4). Preliminary studies by Potter have shown that when a mixture of pyruvate and fumarate was used as a substrate with isotonic KCl tumor homogenates, the addition of sodium fluoride permitted the demonstration of steady rates of oxidation for 20–40 minutes in reaction systems otherwise the same as herein described. The maximum rates were essentially independent of the fluoride concentration, but the maintenance of the rate increased with increasing fluoride concentration up to M/75. On the scale shown in Chart 1, 100-mg. quantities of Flexner-Jobling tumor homogenates gave rates of 15–20 μl. of O_2 per 10 minutes, which correspond to Q_{O_2} values of about 6–9, approximately the rate of oxygen uptake obtained with tumor slices.

Space does not permit a discussion of the various lines of evidence that show a decreased amount of various oxidative enzymes in tumor tissues as compared to brain, liver, kidney, and heart tissues, which in slices show Q_{O_2} values of a magnitude similar to that seen in tumor slices. These tissues may be differentiated from tumors on the basis of a variety of enzyme measurements. However, a number of other tissues, such as spleen, lung, and thymus, have amounts of these oxidative enzymes which do not differ markedly from levels found in tumor. It is therefore of considerable interest that these tissues are sharply differentiated from tumor tissues when tested for their ability to accumulate citric acid *in vivo* following the injec-

[3] Potter, V. R.; Watson, L. S.; and Heidelberger, C. The Oxidation of Labeled Pyruvate and Acetate in Tumor Tissue, in manuscript.

tion of fluoroacetate (15). We believe that the comparison between slices and optimally supplemented homogenates may be interpreted to mean that the oxidative enzymes in the tumor slice must be operating at near capacity, while the slices of normal tissues high in oxidative enzymes are not operating at capacity (cf. 21). This suggests that the oxidative enzymes in the tumor slice may operate at higher concentrations of substrate than the corresponding enzymes in the normal tissues. Whether significant and unique alterations in metabolic patterns can result from such differences remains to be demonstrated, but the possibility is worthy of serious consideration (16).

SUMMARY

1. The oxidation of oxalacetic acid was studied in isotonic homogenates of rat kidney, brain, heart, liver, and transplantable tumors. Analytical studies were carried out to correlate the changes in the concentration of ketoacid, malate, citrate, inorganic phosphate, and ammonia with changes in the rate of oxygen uptake.

2. In each tissue, the appearance of free ammonia was secondary to the output of inorganic phosphate from the adenosine triphosphate reservoir, and the latter was correlated with the failure of the oxidative process.

3. The demonstration of oxidative enzyme activity in the Krebs cycle in the reaction system employed was considered to depend upon a *balance* between the formation of high energy phosphate by the oxidative enzymes and the destruction of high energy phosphate bonds by various phosphatases. It was shown that in the Flexner-Jobling carcinoma and the Walker 256 carcinosarcoma the rate of phosphate breakdown completely overbalanced the rate of oxidative phosphorylation so that no significant ketoacid removal occurred after 10 minutes.

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Histological and Chemical Alterations Produced in Mouse Epidermis by Soft X-Radiation*

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No observations have been reported on chemical alterations caused by soft x-radiation in the epidermis of mice, primarily because there was no method available prior to 1942 (2) for the separation of epidermis from the dermis. Furthermore, the methods for the chemical determination of calcium, total lipids, iodine number of the latter, and the polarographic determination of a reducible substance in lipids have only been recently elaborated by Carruthers, Suntzeff, and their associates (5–7).

Finally, Chaoul's discovery of a contact technic for producing superficial skin lesions by utilizing comparatively soft x-rays, small focus-skin distance, and small irradiation fields has only been recently devised (8). This method made possible limitation of the effects of soft and constant x-radiation to the more or less superficial areas of skin with minimum damage to deep-seated regions (15).

It is important to discover the nature of the chemical alterations due to x-ray, since it is desirable to contrast the action of this physical agent with that of chemical agents like methylcholanthrene. Such a study may well reveal some changes common to epidermal carcinogenesis produced by both a physical and chemical carcinogen and is therefore likely to be basic in nature.

The opportunities to make this comparison were favorable, because essential cooperation on the physical side was available in the Mallinckrodt Institute of Radiology and on the chemical side in the Division of Cancer Research—both at Washington University School of Medicine.[1]

MATERIAL AND METHODS

Radiation delivered by a Phillips Contact-Cavity therapy apparatus was selected, since this was the only means by which a surface tissue could be selectively irradiated. The time required for the treatment was advantageously short, lasting from a few seconds to 1–2 minutes. The only defect thus far noticed was the difference between dosages delivered at the edges of the field and at their centers where small areas of central necrosis developed.

Each animal was immobilized on a wooden frame, and the skin of its back was kept in contact with the x-ray tube during exposure. The object was to irradiate the epidermis only and to avoid the deeper structures as far as possible. A single dose of 3,000 r, delivered to 2.2 cm.^{2} of freshly clipped dorsal skin of 4–5-month-old adult Swiss Albino mice, produced maximal epidermal hyperplasia 21 days later. The source of radiation was 50 kv, 2 ma, 100 r per second without a filter. Two hundred mice of the same age and strain were exposed to this dose in groups of 50. The animals were sacrificed 21 days after irradiation.

For histological examination, skin samples of 30 animals from all groups were fixed in Bouin's fluid. Paraffin sections were cut at 5 μ and were stained with hematoxylin and eosin. A statistical study of the cell types was made by carefully counting cells of different layers per unit area. The cells which maintained some contact to the membrane between dermis and epidermis were listed as basal cells. Those lying distal to the basal cells, and without keratohyaline granules, were counted as spinous cells, and those with keratohyaline granules were classified as granular cells.

From the chemical standpoint, determinations of total calcium, total lipids, iodine number of the latter, and of a reducible substance in the lipids were carried out, because data on these constituents in methylcholanthrene-treated epidermis had been previously obtained. A comparison of the chemical changes in both types of treated epidermis seemed worth-while, since the technics were available. The analyses were carried out as follows:

Total calcium.—Epidermis was separated from the dermis by a method devised by Baumberger, Suntzeff, and Cowdry (2). The epidermis from 60 mice was pooled, weighed, and then placed in a silica crucible and completely ashed at 450° C. in

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a muffle furnace. The calcium content of the ash was determined by the method of Lindner and Kirk (13) and of Solomon, Gabrio, and Smith (22).

Total lipids and iodine number.—For the estimation of total lipids and the iodine number, two samples of epidermis free of hair each from twenty mice were collected in 250-ml. flasks, which contained a cold mixture of 1 part of redistilled peroxide-free ethyl ether and 3 parts of 95 per cent ethyl alcohol. The lipids were extracted and determined by the procedure used by Suntzeff, Cowdry, and Carruthers (23). The iodine number was determined by the Yasuda method (27) and represents the number of gm. of iodine absorbed per 100 grams of fat.

Polarographic analysis.—The epidermis from 70 mice was pooled, and the total lipids were extracted as described above and subjected to polarographic analysis as previously described (6, 7). The polarographic data are given in Table 3, where the half-wave potentials, characteristic constants of the reducible substance, are given at various pH levels.

OBSERVATIONS

Gross observations and histological modifications are given first, since such a study might make possible the evaluation of chemical changes.

Gross description.—From 5–7 days after exposure the skin began to show signs of epilation, which was complete by 10–14 days. An area of superficial necrosis, about 3–5 mm. square, in the center of the exposed area, appeared in all our animals between 7 and 10 days after irradiation. This necrotic area was completely healed by the 21st day. The necrotic lesion was unavoidable when the optimum dose was used. However, it did not appear in animals that were given smaller doses from the same distance or the same dose from a greater focus-skin distance. In these instances the resulting hyperplasia was not maximal, structures other than epidermis began to show a reaction, and, moreover, the mortality in the animals was high. Therefore, even though this temporary superficial central necrosis was always found after delivery of the optimum dosage by the contact technic, no deaths resulted, and the radiation reaction to this dosage was primarily limited to the epidermis—our main objective.

Histological examination.—Untreated epidermis is made up of two to three layers of comparatively undifferentiated epithelial cells covered by a thin layer of keratin. Cells of the basal layer appear more or less cuboidal in sections, and their nuclei exhibit open chromatin networks. The normal epidermis of mice is described by Snider (21).

Irradiated epidermis, consisting of ten to twelve layers of cells, is illustrated in Figure 1. The amount of keratin is increased. This thickened epidermis penetrated down into the dermis in irregular columns, and a few islands of epidermal cells occurred in the dermis. There was marked diversity in nuclear sizes. Many cells were larger and possessed large vesiculated nuclei, with peripherally marginated chromatin (Fig. 3). These types of cells were most numerous in that part of the spinous zone next to the basal layer. Nucleoli were large and occasionally multiple, and chromatin was marked in the nuclei as well as in the cytoplasm. Intracellular spaces and bridges were prominent.

The basal layer was still limited proximally by a basement membrane, and the cells of this layer were narrow with elongated nuclei. Cells in different phases of mitosis were present (Fig. 2). The marked increase in thickness of the spinous layer was the result of mitosis in the basal layer and of the retention of cells in the spinous layer. The cells in the latter stained less intensely with eosin than did the basal cells. Granular cells were also present in increased numbers, and many of them can be seen in the transitional stages of losing their granules; some having completely lost them are merged in the layer of keratin. However, they still have faintly visible boundaries.

Absence of sebaceous glands and atrophy of hair follicles were evident. Table 1 shows the results of a statistical study of the cell types of the three zones in epidermis treated with soft x-radiation. Figure 3 illustrates a marked increase in number of differentiated cells with a corresponding decrease in number of basal cells in both x-ray and chemically induced hyperplasias.

Analysis of irradiated epidermis.—A comparison of the contents of calcium, total lipids, and of the iodine number of the latter from irradiated epidermis with that of the normal and methylcholanthrene-treated epidermis is shown in Table 2. There was no essential difference in the iodine number, and the amount of total lipids of irradiated and methylcholanthrene-treated epidermis was practically the same, being significantly less than normal in both.

The level of total calcium of the irradiated epidermis is about 30 per cent less than normal, while that of hyperplastic epidermis is about 50 per cent less than normal. However, only one sample of irradiated epidermis was available for analysis, so its significance is difficult to evaluate.

Polarography.—The polarographic characteristics of the reducible substance in the total lipids of the irradiated epidermis, along with that from nor-

mal and methylcholanthrene-treated epidermis, are presented in Table 3. There was not sufficient material for electrolysis at all pH values, but the data indicated no difference between half-wave potentials in unbuffered solution and in buffered solutions at pH 4.40 and at 6.38 of the reducible substance from irradiated epidermis, as compared to that of normal and methylcholanthrene-treated epidermis. Therefore, a comparison of the levels of calcium, total lipids, the iodine number of the latter and of the polarographic characteristics of the reducible substance from methylcholanthrene-treated epidermis showed no essential difference from those of irradiated epidermis.

TABLE 1

CELL TYPES IN 21-DAY X-RAY-INDUCED HYPERPLASIA COMPARED TO HYPERPLASIA INDUCED BY METHYLCHOLANTHRENE (12 PAINTINGS)

			EXPERIMENTAL			
	NORMAL*		Methylcholanthrene* treated, 12 ptgs.		Irradiated, 3,000 r at 12-day intervals	
CELL COUNT	No.	Per cent	No.	Per cent	No.	Per cent
Basal cells (B)	988	72.4	912	20.3	694	18.2
Spinous cells (S)	279	20.4	2,930	65.4	2,442	63.9
Granular cells (G)	98	7.18	639	14.3	686	17.9
Total number	1,365		4,881		3,822	
Dividing cells	34	2.49	639	14.3	135	3.4
Degenerating cells	101	7.39	327	7.29	313	8.3
Differentiating cells (S G)	377	27.6	3,569	79.6	3,128	81.8
Ratio of basal cells to differentiated cells	1:0.381		1:3.91		1:5.5	

* Davidhana Banyen (1).

DISCUSSION

Before discussing the results of these observations it is necessary to keep in mind the conditions under which the analyses were made. The chemical determinations were not made on the epidermis from a single animal, but this tissue from a number of mice was pooled so it was not possible to ascertain how closely chemical changes corresponded topographically with the observed histological modifications. Furthermore, the epidermis irradiated by a single dose of 3,000 r after a 21-day interval was used, and it is not known whether this dose is sufficient to induce skin cancer in mice, a well known property of methylcholanthrene.

But reviews of relevant literature indicate that radiation is a potent factor in inducing carcinogenesis. Wetterer's extensive review (24) and observations made by other investigators including Wolbach (25, 26), Colwell (9, 10), Miescher (14), and Bloom (4) support the contention that exposure to radiation by wave lengths shorter than those of the visible spectrum, namely 300 mμ in the ultraviolet or 0.10–0.001 mμ in the x-ray region can produce cancer of the skin (17, 19). Jonkhoff (11) and Sedginidse (20) have investigated the effects of soft x-radiation on mouse skin, and both of these investigators were able to induce cancerous growths in this tissue in a small percentage of their animals. They did not use the above described contact technic. The former employed 100 kv and 50-cm. focus-skin distance, and the latter used 60 kv and a 23-cm. distance.

Data presented here reveal that x-ray and chemically induced hyperplasia of mouse epidermis have much in common, histologically and chemically. The alterations in mouse epidermis are similar to those reported by Snider (21) following beta-radiation and by Miescher (14) after ultraviolet irradiation. It would appear that skin responds in a similar fashion not only to all types of radiations, as reported by Bloom (4), but also to chemical carcinogens.

TABLE 2

TOTAL CALCIUM, TOTAL LIPIDS, AND IODINE NUMBER OF IRRADIATED, NORMAL, AND METHYLCHOLANTHRENE-TREATED EPIDERMIS OF MICE

Treatment	Iodine number (av.)	Total lipid per 100 mg. fat-free epidermis (mg.)	Calcium per 100 mg. of tissue (μg.)
X-ray-treated epidermis	61.20	10.3	30.8
Methylcholanthrene-treated epidermis	61.42*	10.0†	22.2‡
Normal	68.54*	32.2†	43.5‡

* Selna Kaplan, unpublished.
† Suntzeff *et al.* (23).
‡ Carruthers and Suntzeff (5).

Histological changes similar to those reported above were reported by Rhoads (18) and Kidd and Rous (12) in precancerous conditions of skin induced by tars and virus. Large cells with large

vesiculated nuclei having peripherally migrated chromatin have been described by them as characteristic of virus action. These types of cells were also observed in irradiation hyperplasia.

Paletta, Cowdry, and Lischer's (16) histological description of methylcholanthrene-induced hyperplasia is somewhat similar to that observed in x-ray-induced hyperplasia. The histological pictures for these two hyperplasias differ from that of hyperplastic epidermis resulting from healing wounds, as described by Paletta *et al.* (16) as follows:

> Marked variations in sizes of nuclei of basal cells.
> Less extensive intracellular and intercellular edema.
> Less leukocytic infiltration.
> Less affinity of spinous cells for eosin.
> More definite basement membrane.

Thus, the radiation effects herein described are probably not due to an inflammatory reaction.

Many investigators, including Biesele and Cowdry (3) and Kidd and Rous (12), have observed significant changes in nuclei and their component parts during precancerous hyperplasia induced by carcinogens and by tars and a virus. Abnormalities of chromosomes were frequently noticed in cells believed to be neoplastic or preneoplastic. Figure 3 shows similar changes in nuclei after exposure to x-radiation.

Results of the study of the cell types, tabulated in Table 1, also reveal a similarity in response to these heterogeneous stresses. The correlation gives one the impression that the basal cell region is the common target of injuries induced physically as well as chemically. Mouse epidermis under normal conditions possesses 72.4 per cent basal cells and 27.6 per cent differentiated cells. The number of differentiated cells is increased to 79.6 per cent in the chemically induced and 81.8 per cent in x-ray-induced hyperplasia. In other words, these carcinogens perhaps initiate a similar process by stimulating differentiation in the basal cell area. This process of rapid differentiation may be responsible for eliciting the similar histochemical alterations described.

However, a comparison of methylcholanthrene-treated and irradiated epidermis did show some minor differences in numbers of some different cell types. For example, granular cells were slightly more numerous in x-ray-induced hyperplasia than in that caused by methylcholanthrene, while dividing cells were much more numerous in the latter. This may result from a greater mitotic activity in methylcholanthrene-induced hyperplasia than in the x-ray-induced hyperplasia.

TABLE 3

POLAROGRAPHIC CHARACTERIZATION OF REDUCIBLE MATERIAL IN TOTAL LIPID OF IRRADIATED EPIDERMIS COMPARED TO THAT OF NORMAL AND METHYLCHOLANTHRENE-TREATED EPIDERMIS

	Half-wave potential	Unbuffered (volts)*	pH 4.40	5.76	6.38 (Volts)	6.70	7.2
Normal epidermis†	First wave	−1.49	−1.40	−1.49	−1.57	−1.62	
	Second wave	−1.72			−1.65	−1.69	−1.71
Hyperplastic epidermis†	First wave	−1.50	−1.40	−1.51	−1.57		
	Second wave	−1.72			−1.71	−1.70	−1.73
Irradiated epidermis	First wave	−1.52	−1.39		−1.57		
	Second wave	−1.75			−1.66		

* Versus the saturated calomel electrode.
† Carruthers and Suntzeff (6, 7).

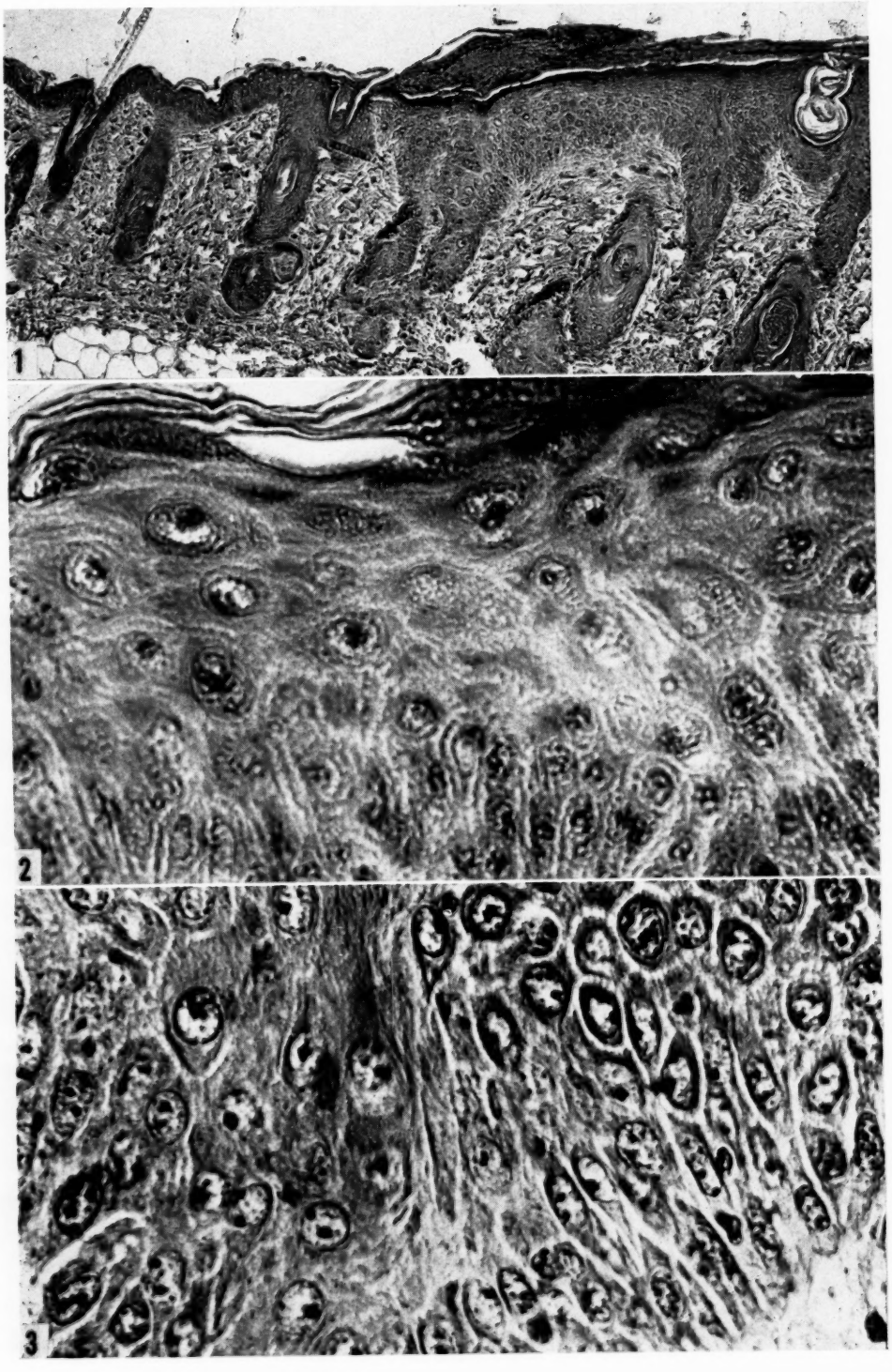
The deficiencies in essential components of epidermis such as calcium and total lipids are remarkably similar in irradiated and chemically induced hyperplasia (Table 2). The level of total calcium of irradiated epidermis is 30 per cent less than normal, while that of the methylcholanthrene-treated is about 50 per cent less than normal.

The total lipids of irradiated as well as of the methylcholanthrene-treated epidermis are practically the same, both being significantly less than normal. There is no difference in their iodine number, as compared to that of lipids from normal epidermis, nor was there any difference detected in a polarographically reducible substance present in the lipids of normal, irradiated, and methylcholanthrene-treated epidermis.

SUMMARY

Areas of epidermis of the backs of adult, female Swiss Albino mice, approximately 4–5 months old, were exposed to soft x-radiation from a Phillips Contact-Cavity therapy machine at a distance of 20 mm. the source of radiation being 50 kv, 2 ma, 100 r per second without a filter. Histological and chemical examinations of the irradiated hyperplastic epidermis were made, and the data so obtained were compared to those relating to normal and methylcholanthrene-treated epidermis.

Histological findings, including the results of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cell type determination, revealed only minor difference between irradiated and methylcholanthrene-treated epidermis. However, total calcium of irradiated epidermis was about 30 per cent less than normal, while that of methylcholanthrene-treated epidermis was about 50 per cent less than normal. No difference was noted in the iodine number of lipids of both, as compared to normal epidermis. The total lipids of the irradiated as well as of methylcholanthrene-treated epidermis were nearly the same in both, being significantly less than normal. A polarographic study indicated no difference in a reducible substance from the lipids of irradiated epidermis (between half-wave potentials of unbuffered solution and in buffered solutions at pH 4.40 and at 6.38) compared to those of normal and methylcholanthrene-induced hyperplastic epidermis.

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FIG. 1.—X-ray hyperplasia of dorsal epidermis of Swiss Albino mouse, 21 days after a single dose of 3,000 r. Hematoxylin and eosin, ×90. Note the junction of normal and irradiated area and compare the thickness of these two regions. The absence of sebaceous glands and atrophy of hair follicles is striking. Keratin layers and spinous zone are markedly increased.

FIG. 2.—X-ray hyperplasia of dorsal epidermis of Swiss Albino mouse, 21 days after a single dose of 3,000 r. Hematoxylin and eosin, ×960. Notice marked thickness of keratin layer. Basal cells have proliferated into many layers of differentiated cells. Epidermis grows into the dermis in a regular way in columns or downgrowths. Sebaceous glands have completely disappeared. Basal cells still maintain contact with basement membrane. Intracellular spaces and bridges are marked. Dividing cells are scattered here and there in various phases of mitosis.

FIG. 3.—Area from same (2). Hematoxylin and eosin, ×960. Notice large cells containing large vesiculated nuclei having peripherally migrated chromatin. Basal cells are narrow with elongated nuclei. Nucleoli are prominent and in many cases are multiple. There is diversity of nuclear sizes.

Studies on a Tumor-retarding Agent Produced by *Aspergillus fumigatus**

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For nearly 4 years a search has been conducted in our laboratories among materials of natural origin for agents having the ability to retard the growth of tumors. Early in the course of this investigation crude culture filtrates of the fungus *Aspergillus fumigatus* were found to retard the growth of the Crocker mouse sarcoma 180 and of the mammary adenocarcinoma E 0771 in mice (4). Meanwhile, Kidd (1) reported that this fungus produced a substance capable of destroying the viability of animal tumor cells *in vitro*. Chemical evidence indicated that this agent was identical with or similar to gliotoxin. Preliminary investigations of the *in vivo* activity of our preparations had indicated that gliotoxin was not the active principle. Thus, studies on the production, extraction, purification, and chemical nature of this new agent were undertaken.

METHODS

All testing was carried out *in vivo* with Sarcoma 180 by the use of an implantation technic (5). Small cubes of 7-day-old tumor, measuring approximately 2 mm. in all dimensions, were implanted, by means of a trocar, subcutaneously into the axillary region of RF or CFW Swiss mice weighing between 18 and 22 gm. Twenty-four hours later administration of the materials under test was begun. Solutions of the active agent were injected intrapertioneally in divided doses over a period of 7 days. Control mice bearing tumors received equivalent amounts of physiological saline. On the eighth day after implantation of the tumor fragments, the tumors on all mice were measured in two diameters by means of calipers. The following arbitrary system of grading was adopted:

Marked inhibition (++).—The average diameters of the tumors in the treated animals were one-quarter or less than that in the control animals.

Good inhibition (+).—Average tumor diameters in treated animals were one-fourth to one-half of those of control tumors.

Slight inhibition (±).—Average tumor diameters in treated animals were one-half to three-fourths of those of control tumors.

No effect (−).—Average tumor diameters in treated animals were three-fourths or greater than those of control tumors.

Each test was run on five animals, five animals serving as controls. Each preparation was tested at least 3 times with consistent results.

Production and extraction of the tumor-retarding agent.—Several strains of *A. fumigatus*, in addition to No. 1 (ATCC 9197) and No. 89[1] (4), were found to produce an agent or agents having the ability to inhibit tumor growth, but for detailed study one strain, namely, 943,[2] was chosen.

Three hundred and fifty ml. portions of a medium, consisting of 2 per cent glucose and 1 per cent Bacto casitone in distilled water, were dispensed in 2-l. Erlenmeyer flasks and sterilized at 120° C. for 10 minutes. The flasks were inoculated with an aqueous suspension of fungus spores and allowed to incubate at 25 ± 1° C. in a stationary state. In several preliminary experiments the maximum production of active material appeared on the seventh to the ninth day of incubation, accompanied by a drop in pH from an initial pH of about 6.6 to pH 3.7–4.2. Further incubation resulted in a rapid loss of activity with a rise in pH. Thus, in the preparation of large lots, cultures were harvested after 7 or 8 days' incubation. The liquid portion was filtered through paper to remove gross cell material. In order to extract the tumor-retarding agent, the crude culture filtrate was saturated with ammonium sulfate at 6° C., as indicated in Chart 1. The resulting insoluble material was collected on a Büchner filter, suspended in 1/40 the original volume of distilled water and

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[1] The authors are indebted to Dr. Kenneth B. Raper for the identification of this culture which in previous publication has been erroneously classified as *Penicillium*.

[2] Dr. Waksman's No. 35. The authors are grateful to Dr. S. A. Waksman for supplying this organism.

dialyzed against running tap water for 2 days and then against frequent changes of distilled water, brought to pH4 with HCl, until the dialysate no longer gave a positive test for sulfate ions. The nondialyzable fraction was filtered through paper. Further purification was accomplished by the addition of an equal volume of cold acetone to a 1 per cent aqueous solution of the $(NH_4)_2SO_4$ precipitated material. The mixture was allowed to stand overnight at 6° C. The resulting insoluble portion was removed by centrifugation, redissolved in a minimum volume of distilled water, and dialyzed to remove residual acetone. The nondialyzable fraction was filtered through paper and lyophilized. This procedure has been used satisfactorily to obtain material from lots of from 1 l. to 22 l. of culture filtrate.

The mycelial pads of the fungus were also found to contain an appreciable amount of a tumor-retarding agent. The pads were ground in a Waring Blendor with 0.1 N HCl (10 ml. per pad). After standing overnight at 6° C., the mixture was filtered and the residue was extracted overnight a second time with the same volume of fresh acid and filtered. The filtrates were combined, adjusted to pH 4 with NaOH, and saturated with $(NH_4)_2SO_4$. The insoluble portion was redissolved, dialyzed, and lyophilized.

RESULTS

A comparison of a preparation from filtrate and one from mycelium is presented in Table 1. Both preparations were carried through $(NH_4)_2SO_4$ precipitation and not further purified. While the weight of material obtained from the crude filtrate was 10 times as great as that extracted from the mycelium, the latter preparation was 10–20 times more potent. Most of the inactive impurities contained in the preparation from the culture filtrate are undoubtedly residual constituents of the medium, since it has been found that Bacto casitone contains a large amount of $(NH_4)_2SO_4$-precipitable material. Whether or not the active principles from the 2 parts of the fungus culture are identical in chemical nature has not yet been ascertained.

Data presented in Table 2 illustrate the effectiveness of an acetone precipitate from culture filtrate. The volumes of the tumors have been calculated on the assumption that the tumor is spherical in shape. It probably approximates more closely an ellipsoid (6), but in these experiments

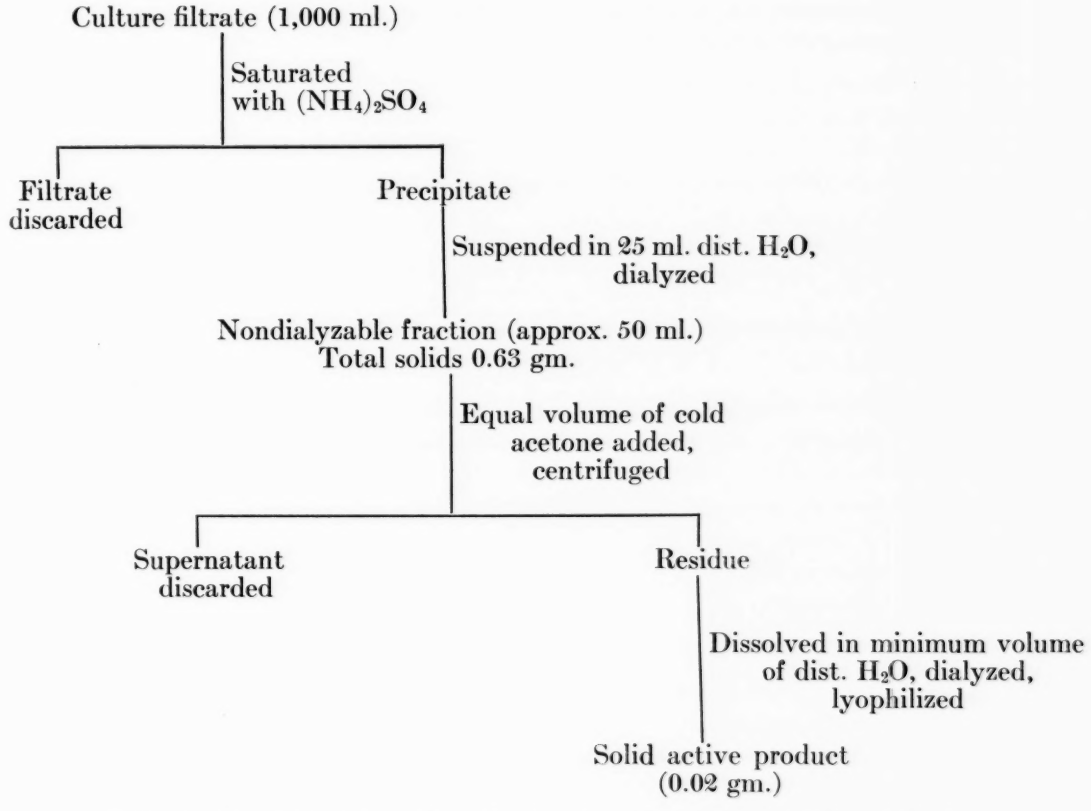

CHART 1.—Extraction and partial purification of tumor-retarding agent from *A. fumigatus*

calculations on this basis are not possible, since the tumors were measured in living intact animals, and data on three dimensions could not be obtained. The intraperitoneal administration of 8.8 mg/kg in saline in divided doses over a period of 1 week was sufficient to cause approximately 90 per cent inhibition of tumor growth. Larger doses resulted in still greater inhibition, while as little as 4.4 mg/kg had an appreciable effect.

TABLE 1

COMPARISON OF TUMOR-RETARDING AGENTS FROM CULTURE FILTRATE AND FROM MYCELIUM OF *Aspergillus fumigatus*

Prep.	Yield per flask (mg.)	Total dose* (mg/kg)	Effect upon Sarcoma 180†
Culture filtrate	160	225	+
		113	±
Mycelium	18	12.5	+
		8.8	±

* Administered intraperitoneally in divided doses for a period of 1 week only.

† +indicates good inhibition, ± slight inhibition. Explained in text under "Methods."

TABLE 2

EFFECT OF DOSE UPON RETARDING OF SARCOMA 180 BY TUMOR-INHIBITING AGENT FROM *Aspergillus fumigatus*

Total dose* (mg/kg)	Average size of tumor† (cc.)	Retardation of tumor growth (per cent)‡	Average weight change in grams per mouse
20.0	0.0021	99	−4.5
12.5	0.0056	98	−3.5
8.8	0.033	90	−2.0
4.4	0.10	70	−1.5
2.2	0.25	30	−0.5
Control	0.36		−0.5

* Administered intraperitoneally in divided doses for a period of 1 week only.

† Volume calculated on the assumption that the tumor is a sphere. $V = 4.189\ r^3$.

‡ Per cent retardation = $\frac{\text{Vol. of control tumor} - \text{Vol. of treated tumor} \times 100}{\text{Vol. of control tumor}}$.

In Table 3 are shown tracings of tumors in mice that received 0.05 mg/day for 5 days, for a total dose of 12.5 mg/kg. On the eighth day after implantation, marked inhibition of tumor development was evident. As has been the case, however, with other agents used in the control of tumor growth such as the folic acid analogs (2), the tumor tissue was not completely destroyed. When treatment was stopped, the tumors proceeded to grow and after 15 days were approximately the same size as the tumors in the untreated control mice.

The ability of preparations from the fungus to retard the growth of Sarcoma 180 has been accompanied by marked general toxicity for the animal, as indicated by the significantly high loss of weight in mice even at dose levels necessary to cause approximately 90 per cent retardation of tumor growth (Table 2). That the inhibitory effects obtained with this agent are not merely the result of malnutrition of the animals has been shown by starvation experiments.[3] Mice bearing Sarcoma 180 were placed on a low caloric diet 1 day after tumor implantation. After 1 week on such a diet the animals lost an average of 4–5 gm. in body weight, but only a slight inhibition of tumor growth occurred. Numerous chemical compounds that have been tested have caused similar losses in weight without having any effect upon tumor growth.

TABLE 3

EFFECT OF 943-375-IBI UPON GROWTH OF SARCOMA 180 IN MICE

SIZE OF TUMORS

TREATED WITH 943-375-IBI*		CONTROLS	
DAYS AFTER IMPLANTATION OF TUMORS 8	15	8	15
-6†	DIED ON 13TH DAY	-1	-3
-2	DIED ON 9TH DAY	-1	-8 DIED ON 15TH DAY
-3.5	-8.5	-0.5	-8.5 DIED ON 15TH DAY
-1.5	-2.5	0	+1
-3.5	-3.5	-0.5	-5

* Prepared by $(NH_4)_2SO_4$ precipitation followed by precipitation with 50 per cent acetone. Each mouse received 0.05 mg/day for the first 5 days after tumor implantation, resulting in a total dose of 12.5 mg/kg.

† Grams change in weight during experiment.

Thus far, efforts to detoxify preparations have resulted in complete loss of ability to inhibit tumor growth.

Detailed pharmacological and pathological studies will be reported elsewhere.[4]

Properties of the tumor-retarding agent.—Unless otherwise indicated, the following studies were made on material extracted from the culture filtrate.

The tumor-retarding agent is soluble in water but not in ether. It is not precipitated from crude culture filtrate by 50 per cent saturation with $(NH_4)_2SO_4$. Although the active principle is readily adsorbed from the culture filtrate by Darco G-

[3] K. Sugiura, personal communication.

[4] J. J. Buckley, S. S. Sternberg, S. Buckley, and F. S. Philips, to be published.

60, attempts to elute it quantitatively from the carbon have been unsuccessful.

Preparations partially purified, i.e., through the precipitation with 50 per cent acetone, have given positive biuret, Millon's, Molisch, ninhydrin, and xanthoproteic reactions.

The tumor-retarding capacities of several preparations have decreased appreciably after treatment with nitrous acid, quinone, and trypsin.

The activity of crude culture filtrates at pH 4 or 7 was completely destroyed by heating in a boiling water bath for 10 minutes. On the other hand, a sterile crude culture filtrate stored at 6° C. for over 15 months appeared to have lost none of its activity. Sterile solutions of an ammonium sulfate precipitate, adjusted to pH's 2, 4, 5.8, and 7.8, were stored at 6°, 25°, and 37° C. After 1 and 7 days, the preparations were tested for activity. The results indicate the following conclusions: (*a*) Storage for 1 week at 6° and at 25° C. at all pH's tested did not appear to destroy the active agent. (*b*) At 37° C. complete inactivation of the active agent occurred at pH 2 after only 1 day and at pH 4 after 1 week, while preparations at pH's 5.8 and 7.8 showed no appreciable loss in potency after 1 week's storage.

In electrophoretic studies the activity originating both from culture filtrate and from the fungus mycelium has been found in a group of highly basic protein components.[5] Although Stedman *et al.* (3) have found that the basic proteins, protamine and histone, retard the development of Carcinoma 2146 in the mouse, protamine sulfate (Armour), when tested as outlined above, brought about no appreciable inhibition of Sarcoma 180, even at a dose that caused the death of 40 per cent of the mice.

In vitro *activity.*—As had been found with crude culture filtrate (4), the $(NH_4)_2SO_4$ precipitate (2 mg/ml) and the more purified acetone precipitate (0.2 mg/ml) from *A. fumigatus* exhibited no demonstrable effect upon Sarcoma 180 *in vitro*. On the other hand, it has been possible in tissue culture to show considerable damage to cells of mouse sarcoma T241 at concentrations that are not toxic to normal tissue. This latter effect has been obtained by the addition of a fresh supply of the active agent to the cultures each day for 4 consecutive days.

[5] M. L. Petermann, M. G. Hamilton, and H. C. Reilly, to be published.

Anti-microbial activity.—The agar-streak method (7) was used to test for possible anti-bacterial and anti-fungal properties of this agent. Crude culture filtrates having very definite effects upon tumor development had no observable effect upon the growth of several gram-positive and gram-negative bacteria.

A purified preparation (of which a total dose of 8.8 mg/kg was sufficient to cause approximately 90 per cent retarding of tumor growth in mice) in a concentration of 1 mg/ml of agar medium had no effect upon the growth of *E. coli*, *B. subtilis*, *B. mycoides*, *S. aureus*, *A. fumigatus*, *A. clavatus*, *Penicillium* sp., *Rhizopus nigricans*, or *Torulopsis utilis*.

SUMMARY

Preparations having the ability to inhibit the growth of Sarcoma 180 in mice have been extracted from the culture filtrate and mycelial pads of *Aspergillus fumigatus*. Chemical and electrophoretic data indicate that the active principle or principles may be basic proteins.

ACKNOWLEDGMENTS

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Fluorescence Studies of Carcinogens in the Rat Skin*

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The resistance of some animal species and strains to the carcinogenic action of synthetic hydrocarbons constitutes one of the most intriguing problems of chemical carcinogenesis. Little morphological and functional evidence has been obtained to furnish satisfactory explanations for various types of resistance to carcinogens involved.

Simpson and Cramer's (25, 26) fluorescence microscopical studies definitely revealed that 20-methylcholanthrene, a powerful carcinogen for the skin of mice upon direct application, was taken up by the sebaceous glands of these animals. They further showed that these glands degenerated and disappeared following one application of the hydrocarbon. Lacassagne and Latarjet (19), Suntzeff, Carruthers, and Cowdry (28), pointed out the involvement of the sebaceous glands in epidermal carcinogenesis. Simpson and Cramer (27), and later Simpson, Carruthers, and Cramer (24), concluded that the sebaceous glands of mice act as a protective mechanism against the carcinogen, and, once destroyed, epidermal carcinogenesis can take place. Therefore, an attempt has been made to trace evidence to explain the relative resistance of rat skin to the local application of two highly potent carcinogens, 20-methylcholanthrene (MC) and 9,10-dimethyl-1,2-benzanthracene (DMBA). A fluorescence microscopical study of this organ after exposure to these hydrocarbons seemed to be indicated for a better evaluation of the fate and the role of the sebaceous glands of this species as compared to those of the mouse.

MATERIALS AND METHODS

Seventy male and female Sprague-Dawley albino rats obtained from a local breeder were used. The average weight of the animals at the onset of the experiments was 110–150 gm., and an older group ranged from 250–300 gm. The interscapular region of the animals was shaved in an area approximating 3 × 6 cm. Two or three brush strokes with a No. 4 camel-hair brush dipped into solutions of 0.6 per cent MC and DMBA in pure thiophene-free benzene were applied to each animal at every painting, according to the standard method used in this laboratory. Four groups, consisting of male and female animals, were studied:

First group.—Normal and benzene-painted controls (20 animals).

Second group.—Single application group. The animals (26) were sacrificed from 10 minutes to 1 week after painting to study the duration of the fluorescence and the elimination of the hydrocarbons from the skin. Four of these animals were in the older age group.

Third group.—Eighteen consecutive paintings (3 times weekly) were applied (12 animals).

Fourth group.—Thirty-six paintings (3 times weekly) were applied (12 animals).

The animals in the latter two groups were sacrificed from 10 minutes up to 4 hours after the last painting.

As hair growth remained vigorous during repeated paintings, especially in the MC groups, the animals were shaved and later clipped regularly with scissors when necessary to secure good contact and spreading of the carcinogen.

Fluorohistological studies of normal and painted skins were made in the following manner: The tissues were fixed in 10 per cent formalin. Then, thick frozen sections (15–25 μ) were washed in water and mounted in a 10 per cent gelatin-glycerin medium to which a few crystals of thymol had been added. This mixture was exposed to daylight before it was used, to reduce the fluorescence of the gelatin and to prevent its interference during the fluorescence studies (12). Nonstained, toluidin-blue-stained, and phosphine-stained sections were studied under visible and ultraviolet light. Frozen sections stained with Sudan IV in an alcoholic solution were used as controls for the studies of the sebaceous glands and the subcutaneous fatty tissue.

The fluorescence outfit comprised a General Electric AH 4 mercury vapor lamp, a Corning filter, No. 5860, and a Zeiss quartz condenser attached to a horizontal microscope containing a

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Wratten 2A gelatin filter in the eyepiece. At first quartz, and later ordinary noncorrosive slides (12, 23), were used for mounting the sections, as they do not interfere with fluorescence studies.

OBSERVATIONS

First group (normal and benzene-painted controls).—A striking sex difference in the normal rat's skin was observed after shaving or clipping when the animal was examined with the naked eye. The soft, pinkish-white skin of the female contrasted sharply against that of the male which was thicker and covered with a coating of brown sebum. This sex difference was not observed in young rats before puberty. To study their normal appearance, the skin sections were examined under ultraviolet light. The findings seemed to be mainly in accordance with those reported in the literature (25, 26). The benzene-painted skins revealed only the fluorescence characteristic of benzene.

Second group (single painting).—Animals killed 5–10 minutes after the application invariably showed the typical blue-violet fluorescence of MC and DMBA in the keratin layer and of the sebaceous glands and ducts. No color differences between these carcinogens could be detected, but the luminescence imparted by DMBA was more brilliant than that of MC.

In thick sections (25 μ), the entire gland fluoresced, like an amorphous luminescent mass. In thinner sections (15 μ), the optically empty nuclei could be detected as black shadows. MC did not penetrate the subcutaneous fatty tissue in male rats (Fig. 1). Only rarely could a few amorphous luminescent globules or granules be seen lying in the dermis adjoining a sebaceous gland acinus. In female rats, however, a blue-violet fluorescence of the subcutaneous fatty tissue could be occasionally observed in some parts of the sections.

DMBA showed the same localizations as MC, but invariably penetrated massively into the subcutaneous fat cells (Fig. 2). Sometimes the hydrocarbon's fluorescence could even be seen in the deep fat cell groups lying between the bundles of the skin muscle. Less penetration was observed in aged animals of 250–300 gm. As a rule, no clearly perceptible blue-violet fluorescence of the epidermal cells could be detected.

The excretion of the hydrocarbons from the sebaceous glands back to the skin surface showed individual variations, also irrespective of sex or the hydrocarbon used. In a female rat this was seen as early as 20 minutes after painting. Excretion of large blue-violet fluorescent drops on the skin surface and sometimes surrounding the free hair-shafts was observed. No blue-violet fluorescence was seen around the hair shafts under the portion into which the sebaceous ducts empty. Between the period of absorption and excretion no fluorescence could be seen for some time in the keratin layer and the sebaceous ducts.

Only traces of the light blue-violet fluorescence of MC and DMBA were observed in some sebaceous glands, and, with DMBA, in some subcutaneous fat cells from 19–24 hours to 4 days after painting. These temporal variations were individual and not related to sex. The carcinogen's fluorescence could be seen mainly in the neck portion of the glands adjoining the beginning of the excretory duct. This slight fluorescence disappeared after ultraviolet irradiation and was not observed 4 days or a week after painting. Usually no fluorescence from the carcinogens or their derivatives could be seen 56 hours after painting.

Toluidin-blue-stained sections exhibited the same colors and fluorescences as unstained sections, except for enhancing the blue color. No mast-cell fluorescence has been observed in any of these sections.

Phosphine-stained sections showed a light purplish fluorescence of the neck portion of the sebaceous glands in the painted skins. Otherwise, they did not reveal anything worthy of mention. All fluorescence was masked by Sudan IV staining.

Painted skins kept for 2 months in formalin revealed no blue-violet fluorescence, either of the sebaceous glands or of the keratin layer, both of which showed their normal auto-fluorescence.

Third group (18 paintings).—Macroscopically, a slight hyperkeratosis, but no hyperemia or thickening of the skin, was noted in the MC-painted animals after repeated paintings. However, the DMBA-painted rats exhibited marked hyperemia and a thickened elevation of the painted skin region which contrasted rather sharply with the bordering nonpainted, pinkish-white or sebum-coated, and unthickened normal skin. Hyperemia persisted for about 24 hours and longer but would then soon subside. MC did not seem to impair the sebum secretion of the male skin, as judged by the persistence of the brown coating. DMBA painting decreased sebum secretion in patches after post-painting hyperemia set in, but a temporary, more active sebum secretion was observed in the regions surrounding the painted skin. Even the hairs proximal to the skin were heavily coated with sebum. The painted regions exhibited a patchy keratinous crust formation.

After repeated paintings in most of the animals, DMBA caused a strong and apparently disagreeable sensation of itching, which occurred simultaneously with the production of hyperemia. Therefore, care was taken that the DMBA-painted rats did not lick off the carcinogen.

Rats that had previously received some applications and then had been killed 10 minutes to 6 hours after another painting, showed a persistence of the sebaceous glands (Fig. 3). The excretion of the painted hydrocarbons to the skin surface was not impaired. No penetration of the subcutaneous fatty tissue by either hydrocarbon (MC, DMBA) was observed, except in the form of amorphous, blue-violet fluorescent drops disposed around some sebaceous glands or hair follicles. The thickened keratin layer in the MC-painted animals showed atypical blue-violet or yellowish-green fluorescence, which was less brilliant than that after a single painting.

The DMBA-painted animals showed a blue-violet or brilliant yellowish-green fluorescence of the keratin or of the keratinous crusts and keratinous plugs closing the opening of the hair shafts. Where such a plug was present, the sebaceous gland of that hair follicle was absent. The sebaceous glands, although persistent, had decreased in size.

No perceptible impairment of hair growth was noted with either carcinogen.

Fourth group (36 paintings).—Every DMBA painting after the eighteenth was followed by a marked hyperemia and thickening of the skin. Judging by the behavior of the rats, the itching sensation was more pronounced. Thick keratinous crusts covered the painted skin regions in patches. These crusts still showed vigorous hair regrowth, but hair growth became partly shaggy. None of these changes were seen in animals treated with MC.

Histological examination showed a persistence of fluorescence in the sebaceous glands in both MC- and DMBA-painted groups (Figs. 4, 5). However, the glands had decreased in number, and none could be seen under the crusts where the hair shafts were plugged by keratin, but they were present in the skin adjoining the crusts (Fig. 5). The degrees of hyperkeratosis in both groups varied individually and regionally. In the MC group small, crusty elevations sometimes could be observed (Fig. 6).

In some sections blue-violet fluorescent droplets were seen to have been artificially shifted from the subcutaneous fat tissue, the sebaceous glands, or the keratin surface into the dermis, as well as into the gelatin-glycerin mounting medium. No significant differences were seen in any of the sections stained with Sudan IV or with phosphine or in those examined for fluorescence.

Since the skin of the monkey is resistant to the action of methylcholanthrene administered topically over a long period of time (22), a fluorohistological study of the skin of this species with methylcholanthrene was carried out. For this purpose, two male and two female rhesus monkeys and one male Java monkey were employed, and the carcinogen was applied as described for the rat. The skin samples were removed 15–30 minutes after a single painting and prepared for fluorescence studies as previously described. The results were, in general, the same as those for the rat, and indicate that the sebaceous gland subcutaneous fat barrier and the sebaceous gland epidermis barrier are important factors in the resistance of the rat and monkey skin to methylcholanthrene.

DISCUSSION

Localization of the carcinogens in the sebaceous glands.—Graffi (17) was the first to use the fluorochrome tracer property of 3,4-benzpyrene for following its distribution after painting on the skin. He localized it in epidermis, hair follicles, sebaceous glands, and the subcutaneous fat. He also observed fluorescent granular storage in the basal epidermal layer after repeated paintings. Mottram and Weigert (21) painted white mice with the same substance and examined their skins by low-power microscopy in ultraviolet light. They observed a blue fluorescence which followed the rows of the hair bulbs and concluded that the carcinogenic derivative of 3,4-benzpyrene was localized in the epidermal Malpighian layer. Later, Doniach, Mottram, and Weigert (15) localized the carcinogen in the sebaceous glands of cleared whole amounts of skin. Independently of these authors, Simpson and Cramer (25, 26) obtained definite evidence for the localization of MC in the sebaceous glands and the subcutaneous fat after skin painting in mice by means of fluorescence studies of frozen transverse sections. In their experiments, the excreted MC-containing sebum made the keratin layer fluoresce blue-violet. Forty-eight hours after the painting of MC, the fluorescence of the sebaceous glands decreased and disappeared 3–4 days after the first painting. The glands decreased in size, collapsed, and disappeared. Blue-violet fluorescent droplets were observed by them during this period in the epidermal layer, especially in the basal cells. This is in agreement with Graffi's (17) findings and those of Ahlström and Berg (1).

These findings in the mouse are in contrast to the observations reported here for the rat. MC enters the sebaceous glands of rats and is excreted by them onto the skin surface, but it penetrates the subcutaneous fatty tissue only in rare in-

stances and in much less appreciable amounts. A physiological barrier seems to exist between the sebaceous glands and the subcutaneous fatty tissue into which they dip during a part of the hair follicular cycle. DMBA, however, penetrates into the fatty tissues of the rat skin as MC does into those of the mouse. The tracer property of these fluorescent hydrocarbons reveals that an active interchange of substances can take place between the sebaceous glands and subcutaneous fat which at times surrounds the glands intimately. It also shows that the interchange with respect to the barrier between these morphological units of the skin may show variations according to both the nature of the painted substance and the species of the painted object. That the physiological barrier against MC in the rat is only a relative one is made clear by the small amount of fluorescent droplets around the glands after painting. These droplets were also observed after 18 and 36 paintings of DMBA, but they were absent in the subcutaneous fat tissues. Whether this tissue resistance to the penetrating action of this carcinogen was built up as an induced barrier, or whether a previously existing physiological barrier became more impenetrable during the relative aging of skin needs further studies.

Size of the sebaceous glands.—It has been observed that the size of the sebaceous glands usually decreases gradually with advancing age. Furthermore, the size of the glands shows variations which are probably cyclic. This is to be expected according to Trotter's findings (29) on hair cycles in humans, and Butcher's (6–8) and Dieke's (13, 14) observations on rats. The studies of Haddow and Rudall (18) also suggest physiological variations in the morphology of the sebaceous glands, since these are closely related to the hair follicular cycle and to hair growth. The observations of these authors may partly explain the difference in size of the glands in the fluorescence microphotographs here presented.

Epidermal and nuclear penetration of carcinogens.—While Simpson and Cramer (25, 26) invariably observed a penetration of MC into the epidermal layers of the skin, such a penetration was rarely observed in the rat. It seemed to be present, however, in the skins after 36 paintings, but its fluorescence was dim and diffuse and not clear-cut. No details, such as the fluorescent droplets in the basal cells of the mouse skin as observed by Graffi (17), Simpson and Cramer (25, 26), and Ahlström and Berg (1), could be detected in rats. Neither was fluorescent material detected in epidermal nuclei, as reported in the literature.

Time of excretion of carcinogens.—This was appreciably shorter in rats than in the mice, as reported by Simpson and Cramer (25, 26). While these authors stated that all MC fluorescence disappeared from all parts of the skin only after 6–10 days, we observed no fluorescence in the rat skin after 4 days.

Persistence of sebaceous glands after repeated paintings.—This was undoubtedly the most striking feature distinguishing the rat skin from that of the mouse in its response to carcinogens. Even after 36 paintings of DMBA only a small number of sebaceous glands were destroyed, while none seemed to be affected by MC. A barrier between these appendages and the subcutaneous fat tissue against DMBA was found to exist at the eighteenth painting, only a few blue-violet droplets being discernible adjoining the sebaceous glands. Furthermore, the glands retained their excretory function throughout the course of the experiment.

It is clear that the sebaceous glands are involved in the absorption, metabolism, and excretion of carcinogens painted on the skin. There are, however, different interpretations as to their role during these processes. Graffi (17) limited himself to a morphological study verifying the concentration of the hydrocarbons in the sebaceous glands. Lacassagne and Latarjet (19) showed that a skin deprived of its sebaceous glands appeared to be refractory to the carcinogenic action of MC, while a skin in which some hair follicles and sebaceous glands were restored after their destruction by irradiation gave rise to rapidly evolving carcinomas.

On the other hand Simpson and Cramer (25–27) concluded that the glands act as a protective mechanism against MC and that epidermal carcinogenesis sets in after their destruction by the carcinogen. They admit, however, that the carcinogen does not penetrate epidermal cells prior to its penetration of the sebaceous glands. Suntzeff, Carruthers, and Cowdry (28) studied the glands in young mice subjected to a single application of MC. They discovered that these were refractory to carcinogenesis although the glands were present. So they concluded: "It would appear that the sebaceous glands, when present, have a conditioning effect upon the epidermis, and that the decreases in the calcium, iron, copper, zinc, and other constituents in hyperplastic epidermis are definitely associated with their disappearance."

Although Dunning, Curtis, and Eisen (16) demonstrated the carcinogenicity of MC in rats subsequent to subcutaneous injection, it is known that this action is rarely manifest when painting of the skin is chosen as method of application. The only

example of carcinogenesis by MC with this method and MC known to us are the four carcinomas produced by Bachmann *et al.* (2). Here, the period of incubation was very long. But, Berenblum (3, 4)[1] was able to obtain benign and malignant tumors by painting DMBA on the skins of rats after long periods of painting and incubation. This carcinogen is defined by a report of the British Empire Cancer Campaign (5) as the "most active carcinogen known."

In conclusion, it was possible to show fluorohistologically that DMBA, the most potent carcinogen for rat skin (3–5),[1] causes the destruction of a small number of sebaceous glands only after repeated applications over a long period of time. MC, a carcinogen practically ineffective for the rat when applied the same way, does not destroy these glands. DMBA penetrated the subcutaneous fatty tissues at the time of the first painting, while MC did not. The times of excretion of both carcinogens in the rat were shorter than that of MC in the mouse. Evidence of fluorescence in the epidermal cells was rare and not clear-cut with either carcinogen. All these factors appear to condition the lower potency of these carcinogens in the rat as compared to the mouse, and the lower carcinogenicity of MC as compared to that of DMBA in the rat. It appears, therefore, that the sebaceous glands and the persistence of their excretory function play the most important role in the so-called susceptibility or resistance toward locally applied chemical carcinogens. The existence of these glands is a prerequisite for the possible chemical induction of epidermal carcinogensis. However, it does not mean that it is the only one. The hydrocarbons are at first concentrated in these glands after their full development. In order to act on the epidermis, they have to cross the physiological barrier between the glands and the epidermis. The existence of a physiological barrier between the glands and the subcutaneous fat could cause the carcinogens to act only locally in case the barrier between glands and epidermal cells was broken down. It would be interesting to ascertain what conditions cause a breakdown of these two barriers in the rat, and their consequences.

In case the sebaceous glands are destroyed, as by MC in mice, the epidermal cells are early sensitized to carcinogenic action and will easily take up the applied carcinogen. In case they are not destroyed, or are only partly destroyed, as in the rat, this sensitization will probably occur much later, and more slowly, and thus the very long periods of incubation may be explained.

[1] I. Berenblum, personal communication, 1949.

SUMMARY

The carcinogens, 20-methylcholanthrene and 9,10-dimethyl-1,2-benzanthracene, were applied in 0.6 per cent benzene solutions to the interscapular shaved and clipped skins of adult Sprague-Dawley rats. Their subsequent distribution was observed by fluorescence microscopy. Both carcinogens enter the sebaceous glands, as Graffi and Simpson and Cramer have reported in mice. However, even after 36 MC paintings, these glands persist in rats, which is in sharp contrast to their destruction after a single painting in mice. DMBA, a more powerful carcinogen for the rat skin, caused limited destruction of the sebaceous glands. The differences between the fate of the two carcinogens in the mentioned two species are discussed as possible factors in their differing response by cancer production. The existence of physiological barriers and their importance in chemical epidermal carcinogenesis in the rat is discussed. Methylcholanthrene was also applied to the skin of five monkeys with results similar to those for the rat.

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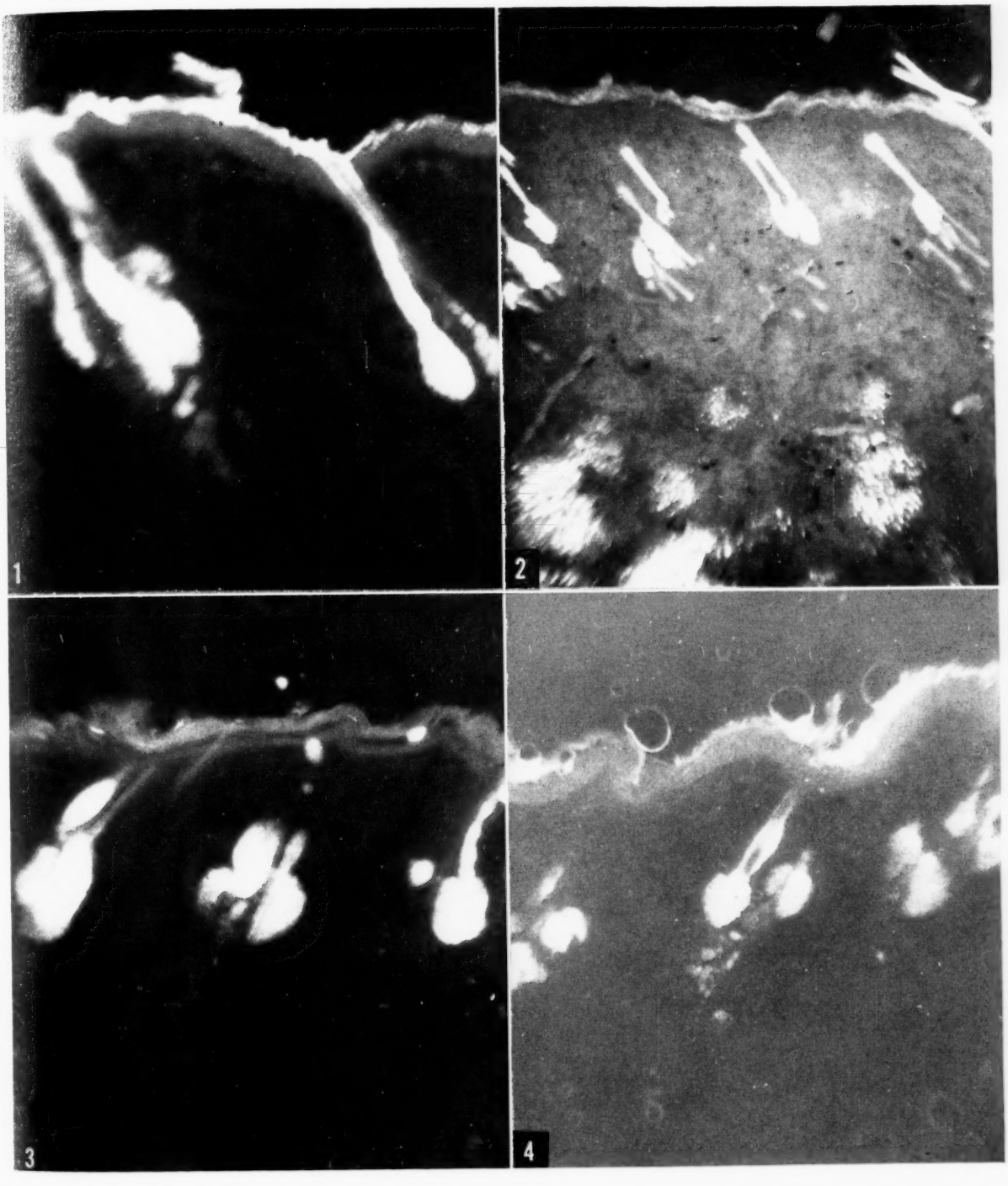
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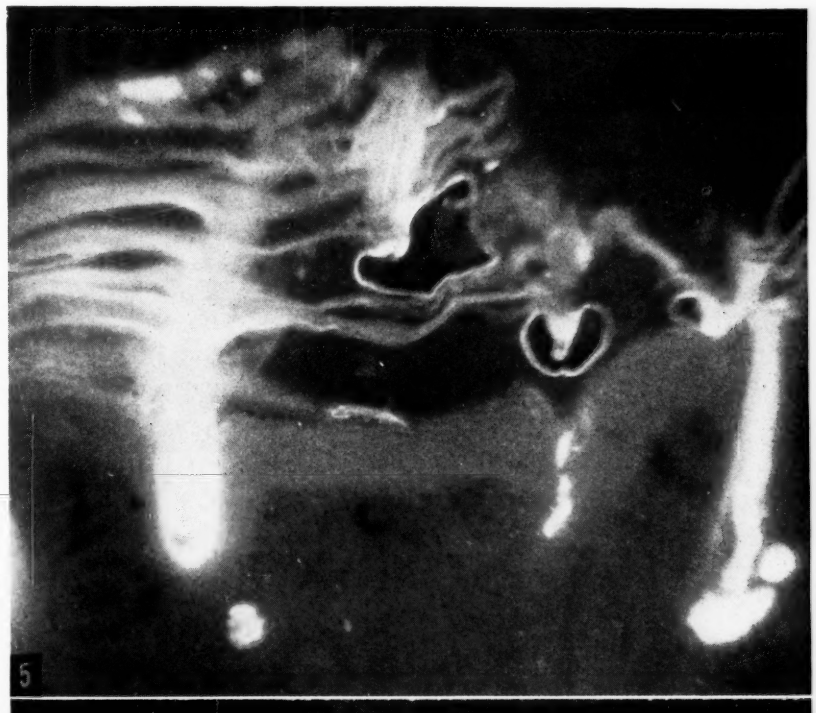
FIG. 1.—Fluorescence photomicrograph of rat's skin 5–10 minutes after a single 20-methylcholanthrene (MC) painting. Mag. ×200.

FIG. 2.—Rat's skin 5–10 minutes after a single 9,10-dimethyl-1,2-benzanthracene (DMBA) painting. The fluorescent carcinogen is seen abundantly in the subcutaneous fatty tissue. Mag. ×160.

FIG. 3.—Rat's skin 10 minutes to 6 hours after 18 paintings with MC. Mag. ×200.

FIG. 4.—Rat's skin 10 minutes to 6 hours after 36 paintings with MC. Mag. ×200.

FIGS. 5 and 6.—Rat's skin 10 minutes to 6 hours after 36 paintings with DMBA and MC, respectively. Fluorescent keratin plugs are seen under the hyperkeratotic layer in Figure 5. Mag. ×200.



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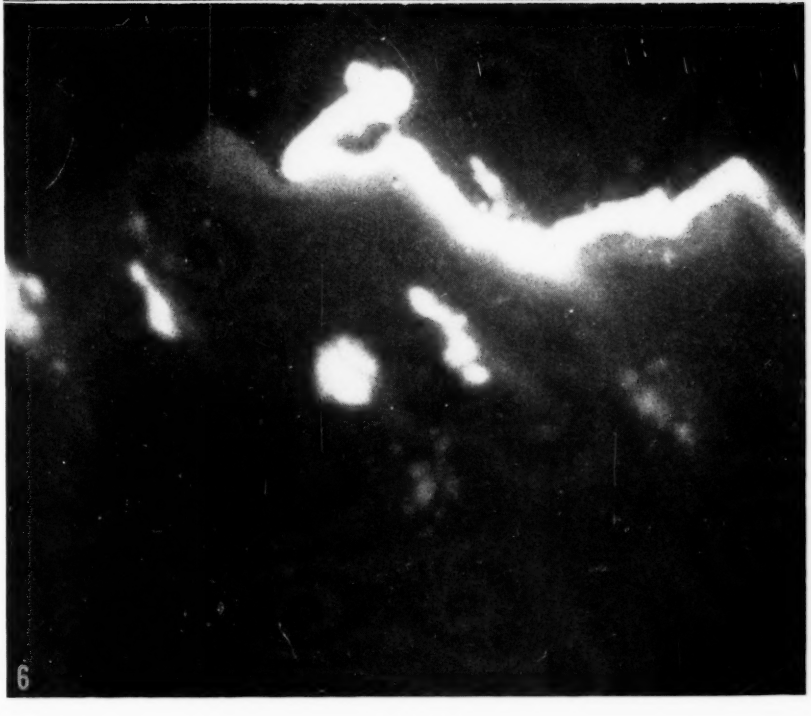

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Electrophoretic and Ultracentrifugal Studies on the Soluble Proteins of Various Tumors and of Livers from Rats Fed 4-Dimethylaminoazobenzene*

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The evidence for an abnormal protein metabolism in carcinogenesis has been reviewed (4, 6, 15, 19). In particular, the studies of Miller and Miller (8, 10) have directed attention to the possible causal role of the protein-bound dye in aminoazo dye carcinogenesis.

The electrophoretic characteristics of the soluble proteins of rabbit liver (16) and the electrophoretic and ultracentrifugal properties of those of rat liver (17) have been reported. In the present study, the soluble proteins of the livers of rats fed either a stock diet or a basal diet, preneoplastic livers of rats fed 4-dimethylaminoazobenzene (DAB), DAB-induced tumors, the liver portions adjacent to these tumors, and separate mesenteric metastases of these tumors have been compared electrophoretically. In addition, an electrophoretic comparison has been made of the soluble proteins of various types of neoplasms, with the results suggesting a common electrophoretic pattern for many tumors. Finally, the soluble proteins of DAB-induced liver tumors, livers adjacent to DAB tumors, and livers of rats fed the basal diet have been analyzed in the ultracentrifuge.

METHODS

Care of animals.—Male Sprague-Dawley rats, weighing 175–200 gm. at the start of the experiments, were housed in screen-bottomed cages and fed, *ad libitum*, distilled water and the semi-synthetic diet No. 3 of Miller *et al.* (9). The diet of the experimental animals contained 0.06 per cent DAB (m.p. 117° C.). In the experiments concerned with preneoplastic liver, the riboflavin administered was 1.6 mg/kg of diet.[1] Since this level was found to yield a slower rate of tumor production than desired, all rats in the reported experiments dealing with DAB-induced tumors received 0.9 mg riboflavin/kg of diet for 20 weeks. At the end of this time, laparotomy of the experimental rats revealed a tumor incidence of close to 100 per cent. All rats were thereafter fed the basal diet containing 2.0 mg riboflavin/kg of diet in order to insure the survival of the animals until the tumors grew to a palpable size.

The Jensen sarcoma,[2] Flexner-Jobling carcinoma,[2] Walker 256 carcinosarcoma,[2] and the chloroform-induced hepatoma 112 B (5)[2] were all obtained from subcutaneous transplants in animals maintained on a stock diet consisting of Purina Dog Chow.

Preparation and analyses of the soluble proteins.—Supernatant fluids of livers and tumors were prepared and analyzed chemically, electrophoretically, and ultracentrifugally, by the previously reported methods (16, 17). With the exception of the preneoplastic DAB experiments, in which the semi-micro-Waring Blendor was used for 2 minutes (16, 17), all tissues studied were homogenized in either a 1×9.25-inch inside diameter or 0.63 × 6-inch (inside diameter) lucite Potter-Elvehjem type homogenizer (12, 13). All but two of the reported electrophoretic determinations were carried out in an 11-ml. cell in veronal buffer, pH 8.6, 0.1 μ at 6.0–6.1 v per centimeter for

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180 minutes (16, 17). Two microelectrophoretic analyses, to be indicated, were conducted in the same buffer with a 2-ml. cell at 8.0 v per centimeter for 80 minutes. The areas were resolved as indicated by the dotted lines of the figures and analytical data for all three experiments are recorded in Table 2. Since similar results were obtained for the three periods, only the representative patterns obtained at 8 weeks are presented in Chart 1. Essentially, the results agree with those

TABLE 1

NITROGEN DISTRIBUTION DATA FOR NEOPLASTIC AND OTHER TISSUES

Tissue	No. animals	No. determinations	Total nitrogen ratios: Supernatant / Homogenate (per cent)	Total nitrogen ratios: Supernatant, dialyzed* / Homogenate (per cent)	NPN in supernatant / Total nitrogen in homogenate (per cent)	NPN in supernatant / Total nitrogen in supernatant (per cent)
Liver of basal diet-fed rats	2	1	53.1	49.3	4.3	7.9
Liver adjacent to DAB liver tumor	5	2	55.2 (54.5–55.9)			
	3	2		52.3 (49.6–54.9)		
	3	1			5.8	10.7
DAB tumor	6	3	62.3 (55.7–70.2)	53.2 (52.0–53.8)		
	3	1			10.4	14.9
DAB metastatic liver tumor	1	1	64.8		12.9	19.9
Jensen sarcoma	2	1	28.1		8.7	32.1
Walker 256 carcinosarcoma	3	1	56.3			
Flexner-Jobling carcinoma	5	1	69.4		8.6	12.4
Tumor 112B	7	1	56.7			
Livers of mice with tumor 112B	7	1	60.2			

*Supernatant dialyzed against sodium phosphate buffer, pH 7.2, 0.1 M phosphate.

TABLE 2

ELECTROPHORETIC DATA OF LIVER SUPERNATANT FLUIDS OF BASAL DIET-FED RATS*

Component	5 weeks: Basal liver (2 rats) Area, per cent	5 weeks: Basal liver (2 rats) Mobility	5 weeks: DAB liver (2 rats) Area, per cent	5 weeks: DAB liver (2 rats) Mobility	8 weeks: Basal liver (2 rats) Area, per cent	8 weeks: Basal liver (2 rats) Mobility	8 weeks: DAB liver (1 rat) Area, per cent	8 weeks: DAB liver (1 rat) Mobility	20 weeks: Basal liver (1 rat) Area, per cent	20 weeks: Basal liver (1 rat) Mobility	20 weeks: DAB liver (1 rat) Area, per cent	20 weeks: DAB liver (1 rat) Mobility
N	6.1	−6.65	3.5	−6.88	2.8	−6.90	1.7	−7.64	3.6	−6.66	5.2	−6.86
A	11.5	−5.74	9.1	−6.03	7.8	−6.09	11.9	−6.44	7.4	−5.79	9.3	−5.92
a_1	26.2	−4.75	35.7	−4.92	39.6	−4.86	41.2	−4.99	42.5	−4.65	36.4	−4.86
a_2	15.4	−3.79	12.5	−3.84	11.8	−3.68	12.3	−3.65	11.6	−3.42	12.1	−3.68
b	12.7	−2.90	11.4	−2.94	12.3	−2.96	9.6	−2.87	9.9	−2.62	10.0	−2.96
g	12.6	−1.99	8.9	−2.09	11.4	−1.99	7.3	−2.02	10.4	−1.73	9.8	−2.08
h_1	7.0	−0.94	8.9	−0.96	7.0	−1.02	7.2	−0.95	6.1	−0.70	6.9	−0.83
h_2	6.7	−0.27	8.4	−0.22	4.9	−0.35	6.4	−0.28	6.0	−0.08	9.1	−0.21
i	1.8	+0.99	1.6	+1.07	2.2	+1.03	2.4	+0.82	2.3	+1.10	1.2	+1.01

*Mobilities have been multiplied by 10^5 cm²/volt sec.

arbitrarily labeled. Protein determinations represent trichloroacetic acid-insoluble nitrogen ×6.25.

RESULTS

NITROGEN DISTRIBUTION FOR VARIOUS FRACTIONS

The extent and nature of the nitrogen fractions extracted from the homogenates of the neoplastic and related tissues are indicated in the data of Table 1.

ELECTROPHORETIC ANALYSES OF THE SOLUBLE TISSUE PROTEINS IN AMINOAZO DYE CARCINOGENESIS

Preneoplastic stage.—The soluble liver proteins of rats fed DAB for 5, 8, and 20 weeks were electrophoretically compared with those of rats fed the basal diet for the same periods of time. The found by use of Waring Blendor-homogenized livers of rats fed a stock diet (Purina Dog Chow) (17). In a subsequent study (18) with 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), fed for 18–19 days, the liver supernatants were essentially similar to the above on electrophoretic analysis. Both the rats fed DAB for 5 weeks and those fed 3′-Me-DAB for 2.5 weeks were sacrificed at the periods of maximum binding by the liver proteins of derivatives of the respective aminoazo dye (8, 10). It is also noteworthy that the liver of the rat fed DAB for 20 weeks was cirrhotic and contained many minute tumors. It is therefore concluded that the gross electrophoretic nature of these preparations of the soluble proteins of

preneoplastic livers of DAB (and 3′-Me-DAB) fed rats does not significantly differ from that of the livers of stock or basal diet-fed rats.

Neoplastic stage.—The liver tumors of rats fed DAB were studied by analyzing, as far as possible, only the non-necrotic portions. These tumors ranged from 0.5 to 5 cm. in diameter. Microscopic examination of the DAB tumor tissue sections consistently revealed a mixture of neoplastic liver and proliferated bile duct cells. Although the least involved portions were selected for study, the livers adjacent to DAB tumors often contained numerous minute tumors too small to remove.

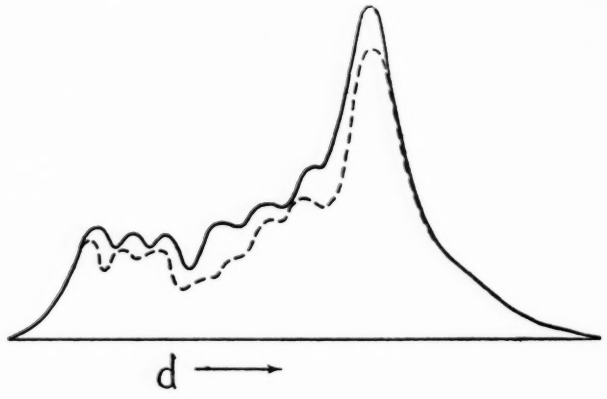

CHART 1.—The electrophoretic pattern of the soluble liver proteins of rats fed DAB for 8 weeks, compared to the same for basal diet-fed rats.
——— = Supernatant fluid of Waring Blendor-homogenized livers of two rats fed the basal diet for 8 weeks; protein concentration, 2–3 per cent.
- - - - = Same for rats fed DAB for 8 weeks; protein concentration, 2–3 per cent.

Electrophoretic analyses have been carried out on the soluble proteins of seven pools of DAB-induced tumors (sixteen rats), the same number of samples of liver tissue adjacent to DAB tumors, and six liver pools from nine rats fed the basal diet. In one experiment, the supernatants of the above were compared with that prepared from multiple DAB-induced liver tumor metastases, each approximately 3 mm. in diameter, taken from the mesentery of one of the previously listed tumor-bearing rats. The analytical data and the representative electrophoretic patterns are presented in Table 3 and Chart 2, respectively. Contamination by minute tumors in the livers adjacent to DAB tumors, homogenized by the Potter-Elvehjem homogenizer, appears to have contributed significant amounts of protein with a mobility range between the b and N components (Chart 2).

TABLE 3

ELECTROPHORETIC DATA OF VARIOUS TUMORS AND RELATED TISSUES*

	DAB											
	Liver tumor (n=7)		Metastatic liver tumor (n=1)	Liver adjacent to tumor (n=5)		Basal diet liver (n=6)		Walker 256 carcinosarcoma Tumor	Flexner-Jobling carcinoma Tumor	Jensen sarcoma Tumor	Hepatoma 112B Tumor	Mouse liver
Components												
	Mobilities × 10^{-5} cm²/volt sec.											
	Av.	S.D.		Av.	S.D.							
P	−8.99	±0.23	−8.83									
O	−7.78	±0.36	−7.74					−7.91	−8.84		−8.19	
N	−6.65	±0.29	−6.48	−6.91	±0.33	−6.86	±0.22	−7.09	−6.78	−6.85	−7.40	−7.57
A	−5.82	±0.28	−5.56	−6.01	±0.33	−5.88	±0.25	−6.30	−5.84	−5.97	−5.87	−6.01
a_1	−4.90	±0.21	−4.64	−4.90	±0.28	−4.83	±0.19	−5.25	−4.67	−4.88	−4.48	−4.90
a_2	−3.92	±0.09	−3.70	−3.89	±0.18	−3.88	±0.14	−4.22	−3.66	−3.93	−3.43	−4.18
b	−2.97	±0.10	−2.82	−3.02	±0.13	−3.02	±0.11	−3.32	−2.92	−3.17	−2.63	−3.38
g	−2.02	±0.12	−1.93	−2.08	±0.17	−2.10	±0.11	−2.42	−2.08	−2.26	−1.77	−2.26
h_1	−1.00	±0.13	−0.84	−0.99	±0.08	−1.06	±0.12	−1.17	−1.10	−1.08	−0.98	−1.25
h_2				−0.34	±0.08	−0.38	±0.15					−0.84
i				+0.99	±0.13	+1.01	±0.13	+0.82			+0.84	+0.60
	Area (per cent)											
P	2.01	±0.4	2.0									
O	2.3	±0.6	3.3					1.7	6.7		1.0	
N	19.5	±3.4	22.4	4.8	±2.5	5.9	±1.5	17.1	12.9	14.0	8.7	3.0
A	20.9	±2.0	20.2	11.4	±2.1	10.0	±1.8	19.1	27.5	24.9	23.7	12.6
a_1	22.4	±3.3	18.1	25.6	±7.6	20.3	±4.6	23.6	25.4	26.8	28.8	27.0
a_2	14.1	±3.1	12.5	19.3	±5.0	17.9	±1.6	15.7	11.2	14.8	14.6	16.0
b	9.9	±1.4	10.8	13.4	±2.3	13.8	±1.7	9.7	7.7	8.0	8.1	14.3
g	6.6	±1.4	7.5	9.9	±1.6	15.0	±2.0	7.6	5.9	8.1	6.3	14.3
h_1	3.1	±0.9	3.2	7.7	±1.5	8.9	±1.8	2.8	2.7	3.5	5.4	4.3
h_2				6.0	±1.2	8.0	±1.4					4.8
i				2.0	±0.5	1.7	±0.2	2.7			3.6	3.8

* Standard deviation, S.D. $= \sqrt{\frac{\Sigma (d^2)}{n-}}$.

However, electrophoretic patterns of the soluble proteins of liver tissue adjacent to DAB tumors microscopically uncontaminated with tumors, have been obtained, which closely duplicate those of the livers of basal or stock diet-fed rats.

To determine the possible influence of the size of the neoplasm on the electrophoretic results, supernatants from DAB tumors less than 1 cm. in diameter, and the non-neoplastic portions of the same livers, were analyzed by use of the micro-

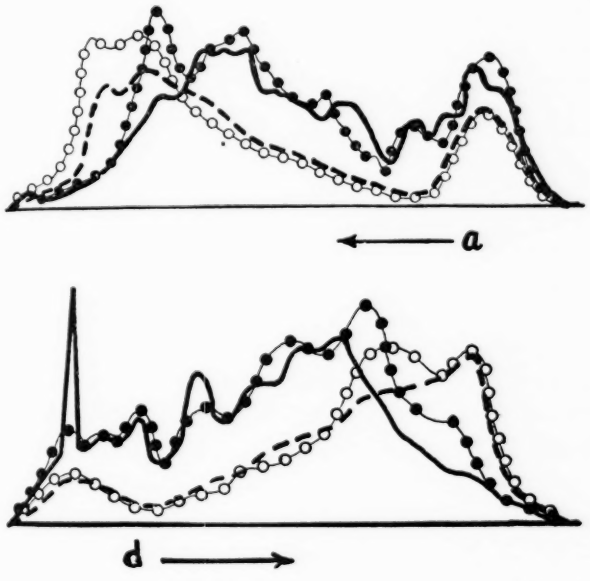

CHART 2.—The electrophoretic nature of the supernatant fluids of the livers of basal diet-fed rats, livers adjacent to DAB tumors, DAB-induced liver tumors, and DAB-induced metastatic mesenteric liver tumor of the rat.
——— = Supernatant fluid of livers of two rats fed basal diet; protein concentration, 2.7 per cent.
●—● = Supernatant fluid of DAB nontumors (three rats); protein concentration, 3.0 per cent.
○—○ = Supernatant fluid of DAB-induced tumor (three rats); protein concentration, 3.1 per cent.
▪ ▪ ▪ = Supernatant fluid of DAB-induced metastatic mesenteric liver tumor (one rat); protein concentration, 2.2 per cent.

electrophoresis cell. The findings agreed with the above results.

It is therefore concluded that under these conditions the electrophoretic properties of the soluble proteins obtained from the livers of rats fed stock or basal diet and liver tissue adjacent to DAB tumors are essentially similar. On the other hand, the supernatant fluids of DAB-induced liver tumor and its metastases are electrophoretically alike. Furthermore, comparison between the two types reveals that the soluble proteins of DAB tumor exhibit a marked reduction of the h (h_1 and h_2) components and large increases in the A and N components.

ELECTROPHORETIC ANALYSES OF THE SOLUBLE PROTEINS OF OTHER TUMORS AND RELATED TISSUES

The above described tissues were also electrophoretically compared with the singly analyzed pools of the following tissues: Jensen sarcoma (2 rats), Flexner-Jobling carcinoma (5 rats), Walker 256 carcinosarcoma (3 rats), chloroform-induced transplantable hepatoma 112 B, and the livers of the same seven Strain A mice. Charts 3–6, respectively, show the electrophoretic tracings of these analyses, while Table 3 contains the calculated areas and mobilities.

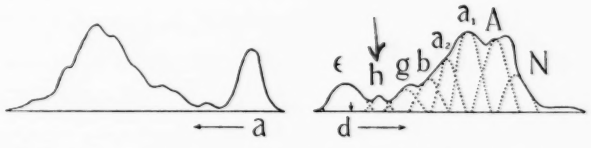

CHART 3.—The electrophoretic nature of the soluble proteins of Jensen sarcoma (two rats); protein concentration, 0.8 per cent.

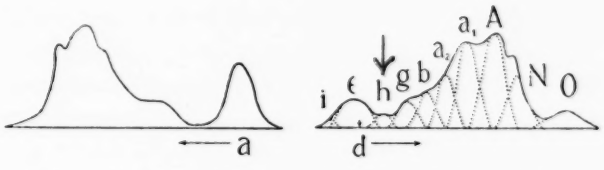

CHART 4.—The electrophoretic nature of the soluble proteins of Flexner-Jobling carcinoma (five rats); protein concentration, 2.8 per cent.

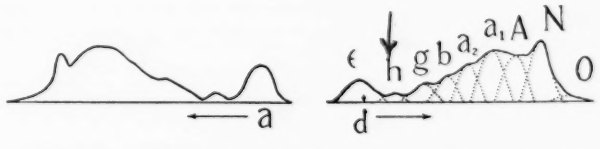

CHART 5.—The electrophoretic nature of the soluble proteins of Walker 256 carcinosarcoma (three rats); protein concentration, 2.1 per cent.

It is evident that, with the exception of tumor 112 B, the electrophoretic patterns of these different tumors resemble those of DAB-induced liver tumor as well as its metastases. It should be noted that these various tumors show a low value for areas analogous to the h components of normal rat liver. Ahead of this region the areas increase as the negative mobility increases, until, in general, a leveling occurs at the a_1, A, and N mobility equivalents. Preliminary studies carried out with 2-acetylaminofluorene (AAF)[2]-induced liver tumors and adjacent liver tissue in general revealed electrophoretic findings similar to those reported for other tumors and non-neoplastic liver tissue, respectively. Mouse tumor 112 B was exceptional, in that protein with the mobilities of the h com-

ponents was present in amounts intermediate between the non-neoplastic and tumor groups. However, compared to the livers of the Strain A mice used for this transplant, tumor 112 B exhibited

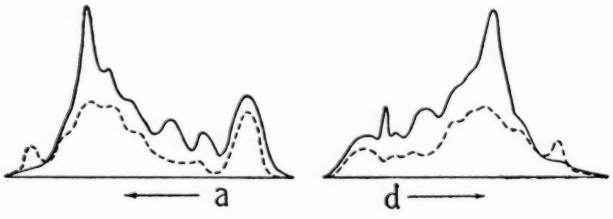

CHART 6.—The electrophoretic nature of the soluble proteins of the chloroform-induced transplantable hepatoma 112 B and the livers of these same seven mice.
——— = Supernatant fluid of the chloroform-induced transplantable hepatoma 112 B (7 strain A mice); protein concentration, 3.2 per cent.
- - - - - = Supernatant fluid of the pooled livers of these seven mice; protein concentration, 3.7 per cent.

(17). In view of the differences in the ultracentrifugal pattern of DAB tumor extracts, as compared to those from extracts of the other tissues referred to above, the tumor components are tentatively designated in the manner shown in Chart 7, in order to indicate the lack of unequivocal evidence at present as to their identity.

Comparison with previous results (17) reveals an essential agreement between the ultracentrifugal nature of the soluble proteins of the livers of stock diet-fed rats, basal diet-fed rats, and liver tissue adjacent to DAB tumors. However, DAB-induced tumor is different, at least insofar as the equivalent regions of the two components, C and D, of DAB tumor are only part of a minor ill-defined group of proteins in the supernatants of the non-neoplastic livers (cf. Figs. 1–3, Table 4, and [17]). Unlike component D, component C flattened early in the sedimentation, apparently

TABLE 4

ULTRACENTRIFUGAL ANALYSES OF SUPERNATANT FLUIDS*

	LIVERS OF BASAL DIET-FED RATS†		LIVERS ADJACENT TO DAB TUMOR		DAB TUMORS		
COMPONENT	S°_{20} S	Area (per cent)	S°_{20} S	Area (per cent)	Component	S°_{20} S	Area (per cent)
I	(69.5)	(5)	(57.8)	(5)	A	(46.4)	(3)
II	46.7	9	45.8	10	B	43.5	9
Minor ill-defined region					C	27.8	9
					D	15.0	9
III	11.6	7	11.9	7	E	10.4	6
IV	6.7	21	6.6	15	F	5.4	11
V	3.3	57	3.8	62	G	3.7	54

* From the livers of basal diet-fed rats (2.8 per cent protein); livers adjacent to DAB tumors (2.8 per cent protein); and their DAB-induced liver tumors (2.2 per cent protein). The numbers in parentheses are for ill-defined components.
† S = Svedberg units, 10^{-13} sec.

elevated A and, to a lesser extent, N components, in agreement with the behavior of the other tumors.

ULTRACENTRIFUGATION OF THE SOLUBLE PROTEINS OF DAB-INDUCED TUMORS AND RELATED TISSUES

Simultaneous electrophoreses of aliquots of the same supernatants which were ultracentrifuged yielded results completely in accord with those presented above. Single sedimentation velocity analyses of the soluble proteins of DAB-induced tumors (three rats), the liver tissue adjacent to these DAB tumors, and the livers of two basal diet-fed rats were compared to those of the livers of rats fed a stock diet (17). Characteristic stages of the sedimentation experiments are shown in Figures 1–3, while Table 4 contains the calculated areas and $s_{20,w}$ values. The designations of the ultracentrifugal components of the supernatants of the livers of rats fed basal diet and the livers adjacent to DAB tumors are the same as previously represented for the livers of rats fed stock diet owing to its greater heterogeneity. Further study is required before significance can be attached to other possible differences among the tissues studied and the livers of stock diet-fed rats (17).

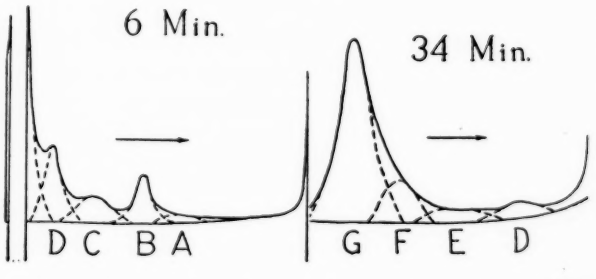

CHART 7.—Tracings of two sedimentation patterns of DAB liver tumor supernatant fluid (duplicate images deleted), showing extrapolated and labeled components. Time at 59,780 r.p.m. indicated.

DISCUSSION

It is apparent that the proteins comprising the h components are present in the supernatant fluids

of livers from rats fed the basal diet, preneoplastic livers of rats fed DAB, and liver tissue adjacent to DAB tumors, but are greatly reduced in those of DAB-induced liver tumor and its metastases. This behavior of the h components may be interpreted as being compatible with a possible protein depletion mechanism of aminoazo dye carcinogenesis, as suggested by Miller and Miller (8, 10). In fact, the distribution of the h components parallels the presence of the protein-bound azo dye in both the preneoplastic livers and the livers adjacent to DAB tumors and in tissue of aminoazo dye-fed rats, and the absence of the protein-bound azo dye in DAB-induced tumors. This correlation is even more strongly supported by the finding, described in the succeeding report (18), that the bulk of the soluble protein-bound azo dye of precancerous livers of aminoazo dye fed-rats is among the proteins of the h components. However, it should be noted that the relatively small amounts of the A and N components in the non-neoplastic livers, and the preponderance of their comparable components in DAB-induced tumor, may be interpreted as being compatible with a possible protein "enrichment" mechanism of aminoazo dye carcinogenesis. At any rate, the over-all increase in the weighted average of the electrophoretic mobilities (in veronal buffer, 0.1 μ, pH 8.6) of the components of DAB tumors, compared to those of non-neoplastic liver, is evidence of a lower weighted average of the isoelectric points of these soluble proteins.

Breedis and Young (3) have reported that liver tumors, including those induced by DAB, are almost exclusively nourished by the arterial blood supply. Since, in the present study, all livers with tumors were perfused through the portal vein, the tumors may have contained some contaminating blood proteins. Elimination of these proteins by the use of arterial perfusion may further reduce the h components.

The ultracentrifugal demonstration that the soluble proteins of DAB-induced tumors contain C and D components is in striking contrast to the finding of minor ill-defined equivalents in extracts of livers from rats fed stock or basal diets, and in liver tissue adjacent to DAB tumors. This difference would agree with an "enrichment" hypothesis of aminoazo dye carcinogenesis. Further study will be required to determine the ultracentrifugal properties of the proteins of the electrophoretic h components.

A most interesting finding is that of the similarity between the electrophoretic behavior of all the tumors studied, with the exception of tumor 112 B. All the former appeared to have only small amounts of proteins of h component mobilities but large amounts of A and N components. Further evidence that this electrophoretic pattern is of even more widespread occurrence among different tumors is available. Miller *et al.* ([11], Fig. 2, *a*) reported an electrophoretic pattern similar to those above for the soluble proteins of mouse rhabdomyosarcoma. A similar pattern for mouse lymphosarcoma was reported by Roberts and White ([14], Fig. 1, 270 minutes). In addition, Barry (2) has recently demonstrated the similar electrophoretic patterns of extracts prepared from rat fibrosarcomas induced by methylcholanthrene and benzpyrene. Furthermore, unpublished studies from this laboratory by Abrams yielded similar patterns for the soluble proteins of a variety of neoplastic lymphoid tissues obtained from humans (1). The similar electrophoretic behavior for the soluble proteins of all these tumors of different "etiology, histogenesis, or even species wherein found" would seem to be consistent with the concept of the chemical similarity of tumors, as expressed by Greenstein (7).

Tumor 112 B was exceptional among those studied by us, in that the amount of protein migrating with the mobilities of the h components appeared to be intermediate between that of normal mouse liver and of the other neoplastic tissues. The relatively slow growth of this tumor[3] (5) may possibly account for this difference. However, it is important to note that low levels of proteins migrating with mobilities of the h components do not appear to be necessarily associated with rapid growth per se, at least in non-neoplastic liver. Thus, the amount of h components in regenerating rat livers, 12 hours, 1, 2, 4, 6, and 8 days after the partial hepatectomy of the main liver lobes, has been found to be essentially the same as that found in the livers of rats fed stock diet.[4]

SUMMARY

1. The soluble proteins of preneoplastic livers of rats fed 4-dimethylaminoazobenzene (DAB) and those of livers adjacent to DAB-induced tumors exhibit essentially the same electrophoretic properties as those of the livers of stock or basal diet-fed rats.

2. The soluble proteins of DAB-induced liver tumor and its mesenteric metastases show a marked reduction of the slowly migrating (vero-

[3] In a personal communication, Dr. A. B. Eschenbrenner, of the National Cancer Institute, stated that tumor 112 B is slow in growth as compared to most transplantable tumors. It requires, on the average, 6 weeks to grow to 1 cm. from an initial subcutaneous inoculum of approximately 2 mm. in diameter, and is nonmetastasizing and only slightly invasive.

[4] Unpublished studies from this laboratory.

nal, 0.1 μ, pH 8.6) electrophoretic h components, and a great increase in the more rapidly moving A and N components.

3. The electrophoretic properties of the soluble proteins of DAB-induced liver tumor, its mesenteric metastasis, Jensen sarcoma, Walker 256 carcinosarcoma, and Flexner-Jobling carcinoma show essentially similar electrophoretic patterns. The slowly growing, chloroform-induced, transplantable mouse hepatoma 112 B yielded an electrophoretic pattern intermediate between those of non-neoplastic mouse liver and other tumors.

4. The soluble proteins of the livers of rats fed stock diet, basal diet, and liver tissue adjacent to DAB tumors exhibit similar velocity sedimentation properties. However, the soluble proteins of DAB-induced liver tumor contain two rapidly sedimenting components whose comparable regions in the non-neoplastic liver extracts are minor and ill-defined.

5. Some implications of these findings with regard to carcinogenesis are discussed.

ACKNOWLEDGMENTS

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Fig. 1.—Selected photographs from the ultracentrifugation of the supernatant fluid from the hepatic tumors of three rats fed DAB; 2.2 per cent protein (N × 6.25); medium, 015 M NaCl, pH 7.2; 50° bar angle; 26° C. The numbers denote the minutes at 59,780 r.p.m. Reference bars have been trimmed off.

Fig. 2.—Selected photographs from the ultracentrifugation of the supernatant fluid of liver adjacent to DAB tumors (three rats), 2.8 per cent protein. Conditions same as in Figure 1.

Fig. 3.—Selected photographs from the ultracentrifugation of the supernatant fluid of the livers of two rats fed the basal diet. Conditions same as in Figure 1.

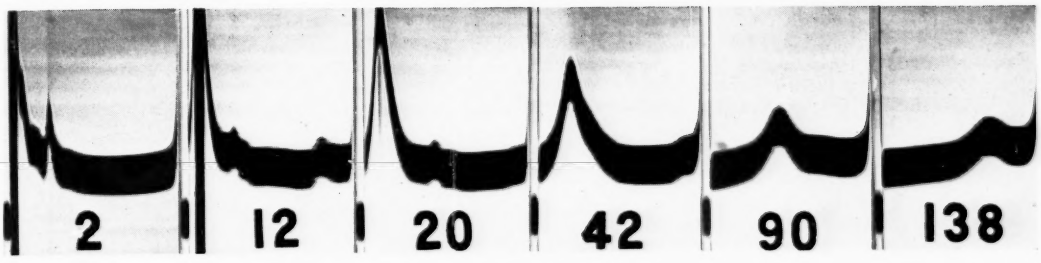

FIG 1

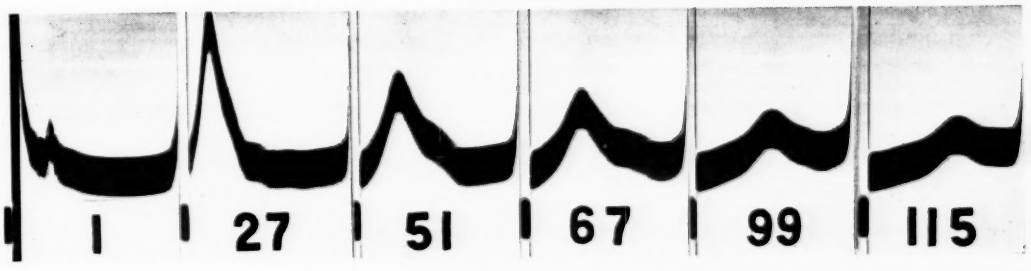

FIG. 2

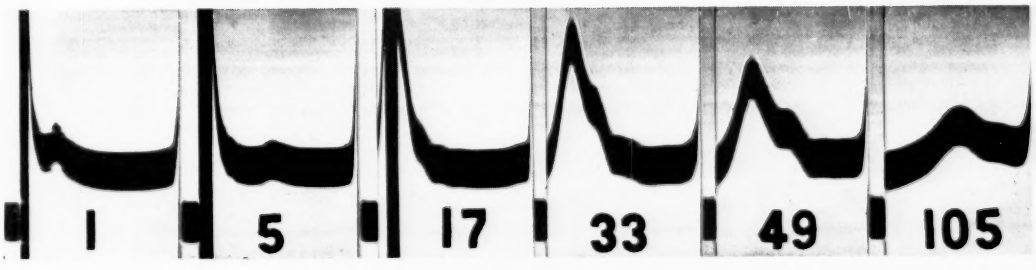

FIG. 3

Electrophoretic Studies on the Soluble Proteins from Livers of Rats Fed Aminoazo Dyes*

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The studies of Miller, Miller, and co-workers (1, 3, 5, 7–10) on the occurrence of protein-bound dye in the livers of rats fed the hepatic carcinogen 4-dimethylaminoazobenzene (DAB) or certain of its derivatives have directed attention to the possible causal role of these proteins in aminoazo dye carcinogenesis. The protein-bound dye appears in the liver a few days after the initiation of dye-feeding and is found in the non-neoplastic portions throughout the stages of tumor formation and growth, as long as the animals are maintained on the dye. However, the protein-bound dye cannot be detected in the aminoazo dye-induced tumors, and this lack can be interpreted as indicating a *qualitative* difference between the proteins of liver and those of the tumors.

Over one-half of the bound dye in these livers is combined with the soluble proteins (7–10). The present investigation was undertaken to ascertain the electrophoretic group(s) of the soluble rat liver proteins which were combined with the dye following the ingestion of aminoazo dyes that have different carcinogenic activities. In previous studies (12, 13), it was found that the soluble proteins of normal rat liver, of livers from rats fed 4-dimethylaminoazobenzene for varying lengths of time short of neoplasia, and of liver tissue adjacent to DAB-induced hepatomas had similar electrophoretic patterns. On the other hand, the soluble proteins of DAB-induced hepatomas, their metastases, and a group of other tumors showed electrophoretic patterns which were very similar to each other but different from these livers. In contrast to the non-neoplastic liver tissues, all the tumors studied exhibited a striking decrease in the slow moving h components and contained more of the A and, to a lesser extent, the N components. It will be shown in the present report that the major portion of the protein-bound azo dye migrates electrophoretically with the same h components (probably h_1) which were previously found to be markedly reduced in the DAB-induced tumor (13).

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METHODS

Male rats of the Sprague-Dawley strain, weighing 175–220 gm., were fed *ad libitum* a semi-synthetic diet (2, diet 3). The aminoazo dyes were incorporated into the diets at a level of 0.058 per cent for 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), and 0.064 per cent for 4′-methyl-4-dimethylaminoazobenzene (4′-Me-DAB). The diet with 3′-Me-DAB contained 1.0 mg riboflavin/kg, while the other two diets contained 2.0 mg/kg. Two rats from each group were killed for each analysis. Two analyses were made on the livers from four rats fed 3′-Me-DAB for 2.5 weeks, two on the livers from four rats fed 4′-Me-DAB for 8 weeks, and one each on the livers of two rats fed 2-Me-DAB for 11, 14, or 16 weeks. At these times the levels of bound dye were approximately at maximum (3). For each analysis the soluble proteins were prepared with a Potter-Elvehjem–type lucite homogenizer and electrophoretically analyzed, by the technics previously described (11–13). After the patterns were photographed, the bottom cell section was closed off to isolate the two vertical limbs. By means of a 5-ml. syringe, equipped with a 15-inch, 20-gauge stainless steel needle and attached to an adjustable screw rack, fractions throughout the entire cell were removed in the order of the Roman numerals shown in Chart 1. The volumes were noted, and the entire samples were transferred quantitatively to tared 15-ml. centrifuge tubes. The protein was precipitated with tri-

chloroacetic acid at a final concentration of 9 per cent. The sedimented protein was washed once with 1 M, pH 5 acetate buffer and extracted 4 times for 5 minutes each with 6 ml. of ethanol at 75° C. After the protein samples were dried *in vacuo* over $CaCl_2$ and finally over H_2SO_4, they were weighed and analyzed for total bound dye (1, 3). Bound dye analyses on similar weights of protein extracted in the above manner agreed closely with analyses on larger samples of the same liver protein, which had been subjected to exhaustive extraction with hot ethanol as previously described (1, 3).

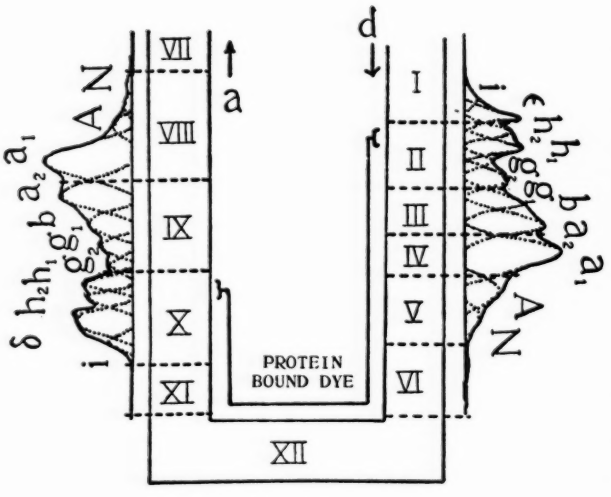

CHART 1.—The typical mapping of an electrophoretic fractionation of the soluble liver proteins from rats fed aminoazo dyes.

RESULTS

As previously found with DAB (13), the soluble liver proteins from rats fed 3′-Me-DAB, 4′-Me-DAB, or 2-Me-DAB for periods of time short of hepatoma development yielded electrophoretic patterns (Table 1) similar to those for normal liver (12). In most cases light yellow boundaries were observed with unfiltered light to move with the h_1 component. In two anomalous runs this light yellow boundary migrated with the h_2 component (see footnote of Table 1). These yellow fronts have not been seen in preparations from rats not fed dye.

In agreement with previously reported results (7–10), all the soluble protein preparations contained high concentrations of bound dye. Chart 1 depicts the mapping of the pipetted electrophoretic fractions, while Table 2 shows the concentrations of total bound dye per milliliter for two representative experiments with 3′-Me-DAB. The dye contents are expressed in E values, i.e., the optical densities of acid solutions of the dyes obtained following extraction of alkaline hydrolyzates of the

TABLE 1

ELECTROPHORETIC ANALYSIS OF THE SOLUBLE LIVER PROTEINS FROM RATS FED AMINOAZO DYES*

Dye fed	3′-Me-DAB	4′-Me-DAB	2-Me-DAB	2-Me-DAB	2-MeD-AB
Weeks fed	2.5	8	11	14	16
No. of rats	4	4	2	2	2
No. of analyses	2	2	1	1	1
		MOBILITIES $\times 10^{-5}$ cm²/volt sec.			
Components					
N	−7.25 (7.13–7.40)	−7.19 (7.16–7.21)	−6.85	−7.06	−7.27
A	−6.23 (6.08–6.30)	−6.04 (6.03–6.05)	−5.90	−6.15	−6.05
a_1	−4.98 (4.95–5.04)	−4.68 (4.61–4.74)	−4.93	−5.10	−4.83
a_2	−3.95 (3.89–4.02)	−3.64 (3.60–3.68)	−3.99	−3.84	−3.74
b	−3.14 (3.06–3.22)	−2.82 (2.76–2.88	−2.96	−2.82	−2.89
g_{g_1}	−2.36 (2.23–2.48)	−2.05 (1.98–2.12)	−2.15	−2.16	−2.05
g_{g_2}	−1.71 (1.63–1.75)		−1.46		−1.27
h_1	−1.00 (0.98–1.02)	−1.12 (0.90–1.33)	−0.86	−0.96	−0.78
h_2	−0.34 (0.26–0.38)†	−0.42 (0.30–0.53)	−0.34	−0.17†	−0.18
i	+0.87 (0.71–0.96)	+1.08 (0.92–1.23)	+0.90	+0.97	+1.09
		AREA (per cent)			
N	6.2 (3.4–9.5)	2.6 (2.0–3.2)	4.7	4.1	3.2
A	9.9 (8.4–11.3)	8.9 (8.1–9.7)	9.5	6.7	9.0
a_1	29.1 (24.0–31.7)	20.8 (20.1–21.5)	19.3	19.4	20.0
a_2	17.5 (16.8–18.9)	19.6 (18.7–20.4)	21.5	25.7	18.2
b	9.5 (8.5–10.2)	12.3 (12.0–12.6)	11.4	9.2	14.8
g_{g_1}	7.3 (6.0–8.3)	12.1 (10.0–14.1)	12.3	14.5	13.5
g_{g_2}	3.7 (2.5–5.0)		3.5		3.6
h_1	7.9 (5.2–10.0)	12.5†	8.2	10.1	8.1
h_2	5.2 (3.9–6.1)†	7.7†	7.3	8.8†	7.6
i	1.7 (1.4–2.1)	2.1 (1.3–2.9)	2.3	1.5	2.0

* Descending electrophoretic data of preneoplastic liver supernatants of rats fed 3′-Me-DAB, 4′-Me-DAB, and 2-Me-DAB.

† These data have been directly affected by a spiking of the descending h_2 component (cf. [1]). Where more than one determination is available, such figures are omitted.

proteins (1). The values E (h_1-corrected)/ml for the border fractions (II and X) were calculated by correcting the concentrations, E/ml, of the bound dye to take into account the dilutions caused by the relative electrophoretic position of the h_1 component within these fractions. This corrected value would be the approximate concentration, E/ml, in the border fractions, if they could be pipetted in such a way as to exclude portions of the cell not containing proteins of the h(h_1) components. It is seen that the protein-bound azo dyes were present at high concentrations in fractions II–VI and X–XII.

Considering the range of E/ml values for the fractions of the cell containing protein without any boundary (fractions VI, XI, and XII) as 100 per cent, the E (h_1-corrected)/ml value of the border fraction (II) can be used to calculate the percentage of the bound dye migrating with the h_1 component. Another method of calculation, by difference, involves the determination of the percentage of the bound dye (E/ml of VI, XI, and XII) which is moving faster than the h($h_1 + h_2$) components on the ascending side (fraction IX). Ignoring the epsilon boundary and minor i component (especially since the yellow boundaries moved with the h_1 component), the remainder is considered to migrate with the h (probably h_1) components. A sample calculation for experiment No. 2 of Table 2 is shown below.

TABLE 2

THE CONCENTRATIONS OF PROTEIN-BOUND DYE IN THE ELECTROPHORETIC FRACTIONS OF THE SOLUBLE LIVER PROTEINS FROM RATS FED 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE*

No.	Units	Fractions I	II	III	IV	V	VI	XII	XI	X	IX	VIII	VII
1	E/ml	0	0.09	0.13	0.16	0.17	0.13	0.18	0.17	0.14	0.04	0.05	0
	$E_{(h_1\text{-corr})}$ /ml	"	0.14	"	"	"	"	"	"	0.16	"		"
	E / 100 mg. protein	0	2.44	1.91	0.98	0.81	0.74	0.75	0.61	0.64	0.26	0.11	0
2	E/ml	0	0.09	0.19	0.18	0.20	0.21	ca. 0.20	0.22	0.13	0.01	0.01	0
	$E_{(h_1\text{-corr})}$ /ml	"	0.15	"	"	"	"	"	"	0.15	"	"	"
	E / 100 mg. protein	0	2.22	2.41	0.97	0.77	0.72	ca. 0.56	0.63	0.60	0.38	0.14	0

* Concentrations of protein-bound azo dye in the pipetted electrophoretic fractions of the soluble liver proteins of rats fed 3′-Me-DAB.

a) Moving *with* h components (calculated as h_1):

$$\frac{\text{E } (h_1\text{-corr}) \text{ / ml of fraction II}}{\text{E/ml of fraction XII}} = \frac{0.15}{0.20} \times 100 = 75 \text{ per cent}.$$

$$\frac{\text{E } (h_1\text{-corr}) \text{ / ml of fraction II}}{\text{E/ml of fraction XI}} = \frac{0.15}{0.22} \times 100 = 68 \text{ per cent}.$$

b) Moving *faster* than h components on ascending side:

$$\frac{\text{E/ml of fraction IX}}{\text{E/ml of fraction XII}} = \frac{0.01}{0.20} \times 100 = 5 \text{ per cent}.$$

$$\frac{\text{E/ml of fraction IX}}{\text{E/ml of fraction XI}} = \frac{0.01}{0.22} \times 100 = 5 \text{ per cent}.$$

On the latter basis, 95 per cent of the total bound dye in the supernatant fluid moved with a mobility slower than that of the electrophoretic components in fraction IX. This slower mobility corresponds to that of the h components, again ignoring the salt and minor i boundaries. Therefore, as the overall range, 68–95 per cent of the protein-bound dye derived from 3′-Me-DAB and contained in the supernatant fluid moved with the h components (probably h_1). Table 3 contains data obtained in a similar manner from the experiments performed with the soluble protein-bound dyes from the livers of rats fed 3′-Me-DAB, 4′-Me-DAB, and 2-Me-DAB.

Similar conclusions are also reached by calculating the data in terms of bound dye per unit weight of protein. As shown in Table 2 for two experiments with 3′-Me-DAB, the proteins in fractions II and III (composed largely of the h components) contained the highest concentrations of bound dye. The values then decreased progressively through the fractions clockwise around the cell, as shown in Chart 1.

Therefore, if the protein-bound dye is consid-

ered to be migrating with the h_1 component, the observed electrophoretic mobility (in veronal buffer, 0.1 μ, at pH 8.6) of the majority of the soluble proteins bearing the dye was -0.98×10^{-5} cm²/volt sec. (average of all analyses in Table 2). The mobility value for the corresponding component in normal rat liver (13) was previously found to be -0.99 ± 0.09[1] $\times 10^{-5}$ cm²/volt sec. If the dye is considered to be carried by the proteins of the two h components ($h_1 + h_2$), the range of similar values would be -0.32 to -0.98×10^{-5} cm²/volt sec (Table 2). The corresponding range of values for normal rats (13) is -0.35 to $-0.99 \pm 0.10 \times 10^{-5}$ cm²/volt sec.

TABLE 3

THE RELATIVE AMOUNTS OF THE SOLUBLE PROTEIN-BOUND DYES MIGRATING WITH THE h COMPONENTS FROM THE LIVERS OF RATS FED AMINOAZO DYES*

Dye fed	Carcinogenicity (4)	Per cent of protein-bound dye migrating with the h components†
3′-Me-DAB	10–12	69–78
		68–95
4′-Me-DAB	<1	61–78
		55–86
2-Me-DAB	0	43–100
		67–100
		46–100

* The per cent of the total soluble protein-bound dye migrating with the h components for rats fed 3′-Me-DAB, 4′-Me-DAB, and 2-Me-DAB in the indicated diets.

† Each range represents the values obtained by the two methods of computation for each experiment. The lower figure was obtained by calculation (*a*) and the higher figure by calculation (*b*) as described in the text.

DISCUSSION

Miller and Miller and associates (1, 3, 6) have suggested that the aminoazo dyes might induce liver tumors by reacting with certain essential liver proteins (for example, those required for the control of growth) in such a manner that cells might arise which lacked these proteins. The lack of protein-bound dye in the tumors produced by the aminoazo dyes was one of the major facts which led to these general considerations. The findings in the present communication are in harmony with this protein depletion mechanism of azo dye carcinogenesis in that the proteins of the electrophoretic group, which include the aminoazo dye-protein(s), are those greatly reduced in amount in liver tumor (12). Of great general interest is the observation that the same components are also low in a variety of other tumors (12).

That the proteins combined with derivatives of 3′-Me-DAB, a very potent carcinogen, have the same electrophoretic mobility as those combined with derivatives of the very weak carcinogenic dyes 4′-Me-DAB and 2-Me-DAB indicates that (*a*) the three aminoazo dyes give rise to the *same* azo dye-containing protein(s) of the h components or (*b*) electrophoresis under these conditions cannot detect the existent differences between the protein(s) bound to the derivatives of these three azo dyes. It is possible that other conditions of electrophoresis or other methods could distinguish between these proteins. In any case, considerable differences in the rate of accumulation of these protein-bound dyes exist *in vivo* and can be correlated with the carcinogenic activities of the parent dyes (3).

The finding that the soluble proteins bound to the azo dyes migrate with the protein(s) of the h components does not necessarily mean that protein(s) of the h components from normal rat liver are bound to the azo dyes. Conceivably, soluble protein(s) of other electrophoretic components, or even insoluble proteins, may act as precursor(s) of these observed aminoazo dye-protein(s) that have the mobility range of the h components. In any case, the finding that the greater portion of the protein-bound dye travels with a small fraction (Table 2; normal rat liver [13]: $h_1 = 8.0$ per cent, and $h_1 + h_2 = 14.7$ per cent) of the total soluble liver proteins suggests that *metabolic derivatives of these aminoazo dyes bind specific protein(s).*

SUMMARY

1. The soluble proteins from the livers of rats fed 3′-methyl-4-dimethylaminoazobenzene, 4′-methyl-4-dimethylaminoazobenzene, or 2-methyl-4-dimethylaminoazobenzene were subjected to electrophoretic analysis. In each case the major portion of the protein-bound dye migrated with the slowly migrating h components. These components were previously shown to be present in greatly reduced quantities in azo dye-induced hepatomas.

2. The results are interpreted to indicate that metabolic derivatives of these aminoazo dyes bind specific liver protein(s).

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[1] Standard deviation $= \sqrt{\frac{\Sigma (d^2)}{n-1}}$.

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Guanine in Cancer*

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It is abundantly clear that the ultimate precursors of the nucleic acids in the Metazoa are to be found among the amino acids. Neither nucleic acids nor purines and pyrimidines are essential nutrients in these species. Whether or not the free bases are intermediates in the biological synthesis of nucleic acids from amino acids is undecided. Adenine alone of the several nitrogenous moieties of nucleic acid is incorporated into the cellular polynucleotides. The incorporation of adenine is more complex than it might appear; when N^{15}-labeled adenine was administered to rats by Brown, Roll, Plentl, and Cavalieri (5) and the nucleic acids subsequently harvested from the tissues, the isotope label was found in the guanine fraction as well as in the adenine fraction. It is probable that the incorporation of adenine in such circumstances is a reflection of a special type of nucleic acid metabolism involving an equilibrium with adenine-containing coenzyme systems rather than an indicator of the steps involved in the biosynthesis of the nucleic acids.

While free adenine is converted in part to polynucleotide-bound guanine, guanine is not utilized at all, according to Brown, Roll, Plentl, and Cavalieri. The latter conclusion was brought into question, however, when Kidder and co-workers (10) discovered the carcinostatic action of guanazolo (8-azaguanine). From the finding that guanazolo inhibits the growth of the protozoan, *Tetrahymena gelii*, for which guanine is a dietary essential, it was suggested that guanazolo is a competitive inhibitor of a unique metabolism of guanine by cancer tissues. Since the compound also inhibits the growth of a number of types of experimental cancer, the assumption was made that neoplasms, unlike normal tissues but like the protozoan, require free guanine for their development.

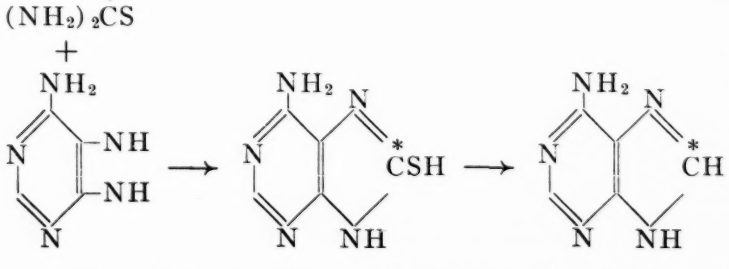
The pertinent question as to whether or not guanine is in fact utilized by cancer tissues was studied by Brown, Bendich, Roll, and Sugiura, (4) but with equivocal results. They reported that mouse tumors did, indeed, incorporate guanine but that rat tumors did not, and, furthermore, that, contrary to earlier observations in the laboratory (5), the normal mouse tissue nucleic acids (black mice) also fixed labeled guanine.

This report describes our investigation of the same problem, in which guanine-8-C^{14} was employed to determine if the purine is incorporated into the cell-bound polynucleotides of either normal tissues or tumor tissues—whether they are susceptible to inhibition by guanazolo or not. It was found that guanine is *not* incorporated in either instance, whereas adenine is readily incorporated into both types of tissue under similar conditions.

Adenine-8-C^{14} in one instance, and guanine-8-C^{14} in another, was administered parenterally to groups of animals bearing spontaneous (virus-induced) breast carcinomas or transplanted breast Carcinoma 755, both of which are readily inhibited by guanazolo. A similar experiment for comparison was carried out on mice bearing Sarcoma 180, which is not inhibited by 8-azaguanine (7). The animals were sacrificed at intervals, and the mixed nucleic acids isolated from the dissected organs were examined radiometrically.

Adenine-8-C^{14} was synthesized according to the scheme:

$$\mathrm{Na\overset{*}{C}N} \longrightarrow \mathrm{Br\overset{*}{C}N} \longrightarrow \mathrm{NH_2\overset{*}{C}N} \longrightarrow \mathrm{NH_2\overset{*}{C}SNH_2}$$



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Guanine-8-C^{14} was prepared similarly by condensing 2,5,6-triamino, 4-pyrimidol with thiourea-C^{14}.

Details of the synthesis of the isotopic purines, the transplantation of tumors, the administration of the isotopic compounds, the isolation of the nucleic acids, and the radiometric comparison are described in the experimental section below.

Thiourea is a versatile intermediate in the synthesis of either purines or pyrimidines, entering into not only the pyrimidine ring but also the imidazole ring. Although the reactions employed in the synthesis of thiourea are familiar, the incorporation of C^{14} involves certain modifications of procedure in order to obtain adequate yields in high purity on a small scale. The method of Baum (2), with some modification, was employed for the conversion of sodium cyanide to cyanogen bromide. Cyanamide was prepared in a manner similar to that of Bloch, Schoenheimer, and Rittenberg (3), except that the ethereal solution of cyanogen bromide, which has an appreciable vapor pressure, was not filtered free of drying agent before treatment with methanolic ammonia. Ammonium sulfide rather than antimony sulfide was used for conversion of cyanamide to thiourea to simplify the purification process.

It is desirable that the isotope be introduced at the latest possible step in the synthesis of adenine-8-C^{14} for reasons of economy. Pyrimidines were first prepared by methods previously reported, and the imidazole moiety of the purine ring was then formed by ring closure involving ortho amino groups and a single carbon atom compound. Traube's (11) condensation of 2-thiol-4,5,6-triaminopyrimidine with formic acid is not feasible for C^{14} syntheses, since a large excess of formic acid is required. For the preparation of adenine containing N^{15} in the pyrimidine ring, Brown *et al.* (5) adapted Baddiley, Lithgoe, and Todd's (1) condensation of 4,5,6-triaminopyrimidine with sodium dithioformate, a compound which is both unstable and difficult to purify. In another modification of that condensation involving the use of a mixture of formic acid and anhydrous formamide, Cavalieri, Tinker, and Bendich (6) reported a 3.8 per cent yield of adenine sulfate-4,6-C^{14}.

The utility and adaptability of thiourea for these syntheses were suggested by the work of Johns (9), who had prepared several 8-thiolpurines by the condensation of molten thiourea with *o*-diaminopyrimidines. Employment of approximately equimolar quantities of thiourea and substituted pyrimidines results in the best yields of 8-thiolpurines, from which the purines can be obtained by desulfurization with hydrogen peroxide.

The ultraviolet absorption in neutral, acid, and alkaline medium served for identification, since the maximum absorption of 8-thioladenine is in the region of 300 mμ, while that of adenine is at 265 mμ. Adenine-8-C^{14} was, therefore, clearly distinguishable from its antecedent, since its absorption curve showed no significant contribution from 8-thioladenine.

EXPERIMENTAL

Cyanogen bromide-C^{14}.—One-half ml. of bromine, 49 mg. (one mM) of sodium cyanide-C^{14}, and 7 ml. of water were stirred magnetically in a 25-ml. distilling flask immersed in ice water. Sodium cyanide solution (100 mg/ml) was added dropwise, until the bromine color disappeared (4.33 ml.). The cyanogen bromide was distilled after 15 minutes into a three-necked 200-ml. flask containing 10 ml. of ether, and cooled in an ice-water bath. To assure complete transfer of the cyanogen bromide, 10 ml. of ether was added to the generator and distilled into the receiver. After being stirred magnetically, the ether layer was removed by a siphon. The extraction was repeated with four 20-ml. portions of ether and the extracts combined and dried over anhydrous sodium sulfate. The yield was determined by treatment of an aliquot of the ether solution with excess standard NaOH solution and back titration with standard HCl solution. It was 84 per cent.

Cyanamide-C^{14}.—Five ml. of 5 per cent methanolic ammonia was added to the ethereal solution of cyanogen bromide-C^{14}. The solution was refrigerated overnight, filtered, and the filtrate evaporated to dryness. The last traces of solvent were removed under vacuum. Crystalline cyanamide-C^{14}, 331 mg., was obtained in 80 per cent yield, based on sodium cyanide.

Thiourea-C^{14}.—Three ml. of concentrated ammonium hydroxide was added to 331 mg. cyanamide-C^{14} and saturated with hydrogen sulfide for 1 hour. The white, pasty cake which formed was allowed to stand overnight, diluted with 10 ml. of water, and boiled until the sulfur agglomerated. The yellow solution was filtered and evaporated to dryness. The residue was leached with boiling water, and the extract decolorized with Norite, evaporated to dryness, and recrystallized from propanol. Thiourea, 587 mg., m.p. 174°–177° C. was obtained in 77.5 per cent yield, based on sodium cyanide.

The specific activity of the thiourea was determined by evaporating an aqueous solution of radioactive thiourea under an infrared lamp after 500-fold dilution with nonradioactive thiourea. The area of the dish was 5 cm.^{2} and contained more than 25 mg/cm^2 of thiourea after being dried to constant weight in a vacuum desiccator. By use of a 1.53 mg/cm^2 mica end window G.M. counter, 1,350 cts/min above background was obtained. The activity of the diluted sample was 68.4 cts/min/mg C, and the specific activity of the undiluted thiourea was 34,200 cts/min/mg C.

8-Thioladenine-8-C^{14}.—Two hundred and twenty-eight mg. (3.0 mM) of thiourea-C^{14} (specific activity 17,100 cts/min/mg C) and 385 mg. (3.08 mM) of 4,5,6-triaminopyrimidine (1) were intimately mixed and heated in a stream of nitrogen at 180° C. The mixture melted and then solidified after 30 minutes. The reaction product was dissolved in hot 2 per cent NH_4OH. The solution was boiled with Darco, filtered, acidified with 6 N acetic acid, and refrigerated overnight. 8-Thioladenine-8-C^{14}, 224 mg., was obtained in a 44 per cent yield. The compound, soluble in dilute alkalies, including ammonia, and in mineral acids, was recrystallized from water.

Calculated for $C_5H_5N_5S$: C, 35.93; H, 2.99; N, 41.91; S, 19.16;

Found: C, 35.97; H, 2.89; N, 41.51; S, 19.37.

8-Thioladenine-8-C^{14} sulfate.—When 143 mg. of 8-thioladenine was dissolved in 14 ml. of hot 1 N sulfuric acid, the sulfate separated on cooling. An elementary analysis indicated the composition to be:

$(C_5H_5N_5S)_2 \cdot H_2SO_4 \cdot 2H_2O$;

Calculated: C, 25.64; H, 3.42; N, 29.91;

Found: C, 25.65; H, 3.57; N, 29.69.

Adenine-8-C^{14} Sulfate.—Two hundred mg. (1.2 mM) of 8-thioladenine-8-C^{14} was treated with 5 ml. of 3 per cent H_2O_2 in a boiling water bath. The solution was filtered after 40 minutes and then cooled in an ice bath for 1½ hours, when crude adenine sulfate precipitated. The first crop of 161 mg. was filtered off, the filtrate treated with platinum black to remove excess H_2O_2, and concentrated for a small second crop. The crude material was decolorized with Darco. The total yield of pure adenine-8-C^{14} sulfate was 174.2 mg. (72 per cent).

Calculated for $(C_5H_5N_5)_2 \cdot H_2SO_2 \cdot 2H_2O$: C, 29.70; H, 3.96; N, 34.65;

Found: C, 29.67; H, 3.95; N, 34.52.

The specific activity was 3,300 cts/min/mg C.

8-Thiolguanine-8-C^{14}.—Two hundred and forty-eight mg. (3.26 mM) of thiourea-C^{14} and 460 mg. (3.26 mM) of 2,5,6-triamino-4-pyrimidol were mixed intimately in a reaction tube. The tube was flushed with a slow stream of nitrogen and heated to a temperature of 180° C. About 5 minutes after reaching this temperature, the reaction mixture became semi-fluid and did not resolidify at this temperature until 25 or 30 minutes later. After 1 hour of heating, the contents of the tube were washed out with hot dilute alkali and filtered. The filtrate, including hot water washings of the residue on the filter, was acidified with 3 N acetic acid. The light brown precipitate which formed was collected in a centrifuge tube, washed with water, alcohol, and ether, and then dried *in vacuo* over P_2O_5. The yield of crude thiolguanine-8-C^{14} was 464 mg. (77.8 per cent).

Guanine-8-C^{14}.—Ten ml. of a fresh 3 per cent solution of hydrogen peroxide was added to 460 mg. of 8-thiolguanine-8-C^{14} in a small Erlenmeyer flask which was immersed in a boiling water bath for ½ hour. An additional 2 ml. of hydrogen peroxide was then added, and heating continued for another half hour. The solution was brought to a boil and filtered after the addition of 1 ml. 10 N sulfuric acid. The red insoluble residue was washed with hot 0.1 N sulfuric acid and with water. The hot combined filtrate (75 ml.) was made alkaline with concentrated ammonium hydroxide. Crude guanine-8-C^{14} precipitated. The yield was 294 mg. (77.5 per cent).

Guanine-8-C^{14} sulfate.—The crude guanine-8-C^{14} (294 mg.) was dissolved in 30–35 ml. of 0.2 N sulfuric acid and heated under reflux with a small amount of Darco for 10 hours, filtered hot, and the residue in the funnel washed with hot 0.2 N sulfuric acid. Pure guanine-8-C^{14} sulfate separated from the filtrate on cooling. The yield was 304 mg.; the specific activity was 3,200 cts/min/mg C.

The tumors used in these experiments were the spontaneous mammary carcinoma (virus-induced) in our own highly inbred Paris R III strain (8), transplanted Adenocarcinoma 755 in our highly inbred C57 strain, and Sarcoma 180, also transplanted in C57 mice. The spontaneous breast carcinoma mice were selected females with well developed tumors, relatively firm and without visible evidence of necrosis. This tumor is susceptible to guanazolo. Adenocarcinoma 755, originally received from Bagg and also sensitive to guanazolo, has been transmitted through 50 passages in this laboratory. It is particularly useful for isotope incorporation experiments, since it grows with fair regularity and does not become necrotic early. Sarcoma 180 has been maintained in this laboratory since 1914. It grows rapidly and is distinctive for its complete resistance to 8-azaguanine.

One or more young, vigorously growing tumors was dissected under nearly sterile conditions, and small fragments were introduced by trocar intramuscularly in the flank into 48 female recipients. The tumors took in all cases and were allowed to develop until they had attained a size approximating 1 gm., but without visible necrosis. The 755 tumor grew to this convenient size in about 14 days; Sarcoma 180 required about 10 days. Half of each group, 24, were given 1 ml. each of a solution containing 0.5 mg. adenine-8-C^{14} sulfate intraperitoneally; the remaining 24 received 1 ml. of a slightly alkaline solution containing 0.5 mg. guanine-8-C^{14} sulfate. Eight animals from each group were sacrificed 6 hours later, eight more in 24 hours, and the remaining eight at 30 hours. Tumors, livers, kidneys, and small intestines were dissected out and pooled. The nucleic acids were isolated from these pooled organs.

Fractionation and isolation of the *individual* nucleic acids will be discussed in a forthcoming publication. The immediate purposes of this experiment, however, were served by the isolation of the mixed nucleic acids without partition of the pentose and desoxypentose types. Thus, specimens prepared from different tissues contained different proportions of the two or more nucleic acids. Preparation of the mixed nucleic acids consisted of simple expedients to dissociate possible protein-nucleic acid linkages and to salt out or otherwise denature the proteins, while retaining the nucleic acids in solution.

The freshly harvested tissues were dispersed in 10 volumes of cold saturated NaCl solution with additional solid NaCl approximating the tissue in weight. A Waring Blendor adapted to small volumes was used. The viscous, creamy suspension formed was stirred vigorously by motor in the cold for 1 hour. Small portions of ether were then added to the cold suspension at intervals until the ether phase exhibited a tendency to separate quickly when stirring was stopped. After stirring for about ½ hour more, the ether-salt-tissue suspension was centrifuged at room temperature for 15 minutes at 3,000 r.p.m. Three distinct, separable phases were formed: the bottom layer, a solid phase composed

of salt and insoluble tissue components; the middle layer, a saturated solution of the nucleic acids and a number of other solutes; and the uppermost layer, an ether-protein gel. The middle layer was most conveniently removed by a syringe and large-bore needle and clarified by centrifugation in the Sorval SS1 centrifuge at 10,000 r.p.m. for 15 minutes. The water-clear supernatant solution was ejected into 3 volumes of 95 per cent alcohol. The nucleic acid salt which precipitated was collected on a Büchner funnel and washed free of water with 95 per cent alcohol, and then with ether, and allowed to dry in air. The mass of salt and nucleic acid was dissolved in water with the aid of the Waring Blendor, and the salt was then removed from the solution by rapid dialysis with tube-shaped collodion bags, each containing a large glass bead. The contents of the bag were agitated by end-over-end rotation of the bag in a large volume of ice water. Almost all the salt was removed in about $\frac{1}{2}$ hour by vigorous agitation and three changes of water. The solution in the bags was transferred to pyroxylin tubes and recentrifuged at 10,000 r.p.m. in the Sorval for 10 minutes before reprecipitation in 3 volumes of alcohol. This precipitate was most conveniently collected and dehydrated in the centrifuge tube. The preparations were adequate for the purpose when they dissolved readily and completely in the 1 per cent sodium chloride solution and when the absorption curve coincided with that of nucleic acid.

"Infinite" thickness specimens of the sodium nucleate preparations were measured under a mica-window Geiger-Müller counter. The counts observed have only approximate significance, since particle size and distribution were not ideally uniform in these specimens. The data for adenine are recorded in ratio of specimen radiation to background radiation (Table 1). The data for guanine are not recorded in the table, *since in no instance was any radiation significantly above background detected.* Sufficient counts to produce a statistical precision of 5 per cent were taken. The background varied between 0.30 and 0.40 counts per second.

DISCUSSION

The data show that cancer tissues do not uniquely utilize guanine in the synthesis of polynucleotides, and there is no evidence that the biosynthesis of nucleic acids in tumors is distinguishable from that in normal tissues which do incorporate adenine but not guanine. Carcinostasis by 8-azaguanine does not involve synthesis of nucleic acid from guanine.

The test compounds employed to demonstrate these facts were administered intraperitoneally, because nucleic acids, purines, or pyrimidines are not essential to the mouse diet, and, if the purines were to be intermediary synthetic metabolites, it seems probable they would be formed in close proximity to or within the cell rather than be transferred from or formed within the alimentary tract. That the guanine so administered did reach the vicinity of the cells is indicated by the fact that alcohol-dehydrated organs from similarly treated animals were radioactive. This radioactivity could be decreased by extraction of the tissue powder with cold trichloroacetic acid. The nucleic acids subsequently isolated from the tissue powder, however, were devoid of radioactivity. It seems probable that minute deposits of guanine-8-C^{14} or xanthine-8-C^{14} were the source of the whole tissue radioactivity. Indeed, in another experiment in which very large doses of guanine-8-C^{14} were administered (4 mg. per day in divided doses for 3 days), multiple plaques of practically pure guanine were visible in the viscera. The organs of these animals were highly radioactive, and the radioactivity was leached out by cold trichloroacetic acid only slowly and with difficulty. As before, the nucleic acids exhibited no significant radioactivity above background. The resistance to trichloroacetic acid extraction suggests that, had the purines rather than nucleic acids been extracted from the tissue, as is feasible following hydrolysis of untreated tissue, it is probable that opposite and erroneous conclusions would have been reached.

TABLE 1

RATIO OF SPECIMEN RADIATION TO BACKGROUND RADIATION, INFINITE THICKNESS, IN THE NUCLEIC ACIDS ISOLATED AT INTERVALS AFTER THE ADMINISTRATION OF RADIOACTIVE ADENINE

	Hours	755	Spont.	180
Tumor	6	1.8	1.4	1.5
	24	2.2	1.8	1.7
	30	2.4	2.2	2.0
Liver	6	1.4	1.5	1.4
	24	1.7	1.8	1.6
	30	2.0	2.0	2.1
Kidney	6	1.4	1.6	1.5
	24	1.8	1.8	1.9
	30	2.1	2.0	2.1
Small intestine	6	1.8	1.7	1.4
	24	2.0	2.1	1.8
	30	2.1	2.0	2.2

The adenine data, confirming Brown, Roll, Plentl, and Cavalieri's original finding, and extending it to include tumors, are of additional interest in that the rates of incorporation of adenine

in different tumors and different organs approximate one another. This point and its implications will be discussed in a forthcoming report from this laboratory.

SUMMARY

1. Guanine is not incorporated into the cellular polynucleotides of either normal tissues or cancers.

2. Adenine is incorporated into cancer polynucleotides at a rate approximating its incorporation in normal tissue polynucleotides.

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